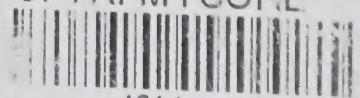


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CYTOLOGY AND
CELL PHYSIOLOGY

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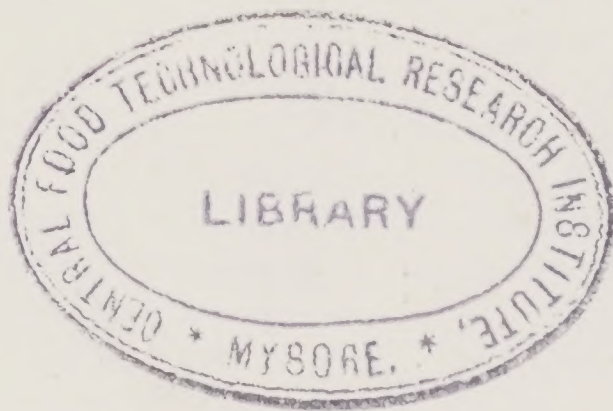
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CYTOLOGY AND CELL PHYSIOLOGY

EDITED BY
GEOFFREY H. BOURNE

SECOND EDITION



OXFORD
AT THE CLARENDON PRESS

'Cytology may rightly claim to be the frontier state in the biological commonwealth, for within its borders biologists and chemists find common ground.'

PROFESSOR JAMES GRAY, 1931

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4314

Cytology and cel.

FOREWORD

DURING an argument a Liberal and a Conservative in the old-fashioned sense discovered to their surprise that there was little in fact to choose between their idealized positions. So it is with the cytologist and biochemist; initially so widely separated in method and outlook, so far as the biochemist's concern is chemistry and life, this is also the ultimate cytological aim. Some years ago the writer of this foreword was forced to urge the need for a bolder and more imaginative co-operation from the standpoint of known biochemical facts about the nucleo-proteins, finding a possible solution of some difficulties about the living cell in a conception which has subsequently been called the 'cyto-skeleton'. Arguments borrowed from organic chemistry will do much to provide a partial explanation of the unity and division of the cell.

This book is wanted to form a permanent record of the tendency for these two previously separated groups of workers to draw together. Most people really waste the time of enforced idleness which is such an inevitable accompaniment of a war. It is greatly to the credit of the contributors here that they have fought mental lethargy to the point of creating this valuable composite statement upon the cell. The spirit is indeed hard to crush; the weakness and strength of the flesh rests upon organized chemistry.

The above words were written in the foreword to the first edition of this book in this month seven years ago. Now that the spirit has indeed triumphed, there has come leisure for this second edition. The very fact that this is wanted speaks in itself for the value of this book. I can commend it sincerely to those who wish to think more deeply about the varied problems presented by the living cell and so to approach a better understanding of the unit upon which the phenomena of life are based.

27 July 1948

R. A. PETERS

PREFACE TO SECOND EDITION

IN the new edition of this book which is published some eight years after the first edition and during a period of peace, the same general form and all the existing chapters have been retained. All the chapters have been revised and rewritten with new material added, and in some cases substantial changes have been made. The importance of the pathological aspect of cytology has made it necessary for this particular chapter to be greatly enlarged. There are two new complete chapters, one on 'Histogenesis in Tissue Culture' by Honor B. Fell, and one on aspects of evolutionary cytology by E. N. Willmer. Two portions of chapters are contributed by F. K. Sanders and R. Barer, who write on 'Special Methods' of cytological investigation and microscopy. Dr. Sanders has also assisted in the revision of Chapter VIII.

I am indebted to the Ministry of Supply by whose kind permission the electron microscope photographs in Plates 3 and 4 illustrating Dr. Barer's article on microscopy are reproduced.

Once again the authors and the Oxford University Press are thanked for their promptness and help in the production of this new edition.

G. H. B.

LONDON HOSPITAL MEDICAL COLLEGE,
WHITECHAPEL, E. 1

PREFACE TO FIRST EDITION

THE phase of purely morphological investigation of cells is now changing into a period in which the interpretation of structure in terms of chemical composition and function is the aim of many cytologists. This does not mean that we have learnt all that the morphologist has to tell us, for there are many problems of cell structure which he has yet to solve. But it means that the morphologist will need to work, not as before in a watertight compartment, nor even in a compartment which is covered with a semi-permeable membrane, but in one which will permit an intimate mixing of his knowledge with that of the physiologist, the biochemist, and the physical chemist: for so complex are cellular organization and function that the brain of no one man can hope to envisage their manifold complications.

In this book an attempt has been made to bring together chemical, physicochemical, and morphological aspects of the study of cells. It has not been the aim to cover the whole field of cytology or of cell chemistry, indeed it would take a series of volumes to do so. The best that one can do is to choose a number of subjects which are representative of different fields of the study of cells and which relate, as far as possible, one to the other, and to bring them together within a single cover.

The problems of producing such a book as this in war-time are not inconsiderable, and the editor wishes to thank the contributors, who are all scientists working in war-time Britain, for the way they overcame their many difficulties and the speed with which they produced their various chapters.

The whole book was written during the course of the 1940-1 air blitzkrieg on Britain, and there are probably no chapters of which part was not written within the sound of bursting bombs. One contributor, in fact, wrote almost his entire chapter by candle-light in an air-raid shelter during the worst bombing attacks on London. Another author wrote his while on sick leave from one of the fighting services, and a third produced his contribution chiefly in railway carriages while travelling from one urgent war duty to another. Most authors have written their chapters in what little time they had left over from war research. Others who have not been occupied directly in this way have had their time severely curtailed by other war duties such as acting as air-raid wardens, &c.

Incidental difficulties have harassed the authors in various ways. Those who were working in areas subject to frequent air attack had to contend with the closing of libraries while air raids were in progress, or with the evacuation of libraries to other parts of the country. The

latter difficulty often resulted in long and tedious journeys to obtain essential periodicals.

Thanks are also due to Professor R. A. Peters for his interest in the subject of this book and for sparing time from his many urgent duties to write a foreword for it.

It is a pleasure to acknowledge the care and attention which the staff of the Oxford University Press have given to the production of the book and to congratulate them on the speed with which, despite their occupation with urgent war work, they were able to place it in print.

G. H. B.

UNIVERSITY LABORATORY

OF PHYSIOLOGY

OXFORD

December 1941

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CHAPTER I

CYTOLOGICAL TECHNIQUES

A. PREPARATION OF TISSUES FOR MICROSCOPICAL EXAMINATION AND HISTOCHEMISTRY

By JOHN R. BAKER

i. INTRODUCTION

THANKS to recent advances in histochemistry, it is nowadays once more possible for the study of substance to go hand in hand with the study of the minute structure of organisms, as they went long ago when the introduction of histological staining had not yet driven a subtle wedge between the two. To-day the cytologist is or must become something of a biochemist, but a biochemist of an unusual sort. It was once remarked to the writer that biochemists like to have their substances in test-tubes. The cytologist wants to have his exactly where they were in life, and to know, as precisely as he can, what they are. When substance and structure are known, the way is clear for the elucidation of the main problem of cytology, which is to discover what a cell does to keep alive and to perform its functions for the body as a whole or for the next generation.

To some extent cytological technique is a craft. No one will succeed in it who finds its processes tedious. To ensure the making of a good cytological preparation, of whatever kind, it is necessary not only to take such precautions as have been proved to be valuable in the particular case in hand, but also to do everything that may reasonably be supposed to help towards the desired end. Often there are many small ways in which one can prevent distortion of cellular detail, and it is not possible to prove conclusively that each one is actually helpful in every case; but if all the small precautions are omitted for the sake of speed or as a result of laziness, success is unlikely.

Time may often be saved and success secured by the adoption of long techniques. In these days, when rush-methods are appreciated beyond their worth, many people are prepared to waste almost any amount of time if only they can get results quickly. The quick methods often take up more of one's time than the slow. The leaving of a slide overnight in a solution occupies none of anyone's time; the holding of it under the same solution over a flame engages someone's close attention for several minutes, and delicate structures may be injured.

It must be allowed that some procedures are unnecessarily tedious. The profusion of different substances used and the complication of

many of the methods often seem out of all proportion to the value of the results obtained. It is a striking experience to make a permanent preparation of a mammalian testis by Benda's hugely elaborate process—involving, as it does, prolonged fixation followed by two separate mordanting processes of the tissue in bulk and two further mordanting processes applied to the sections, and then staining of the mitochondria and a very tricky differentiation, and lastly dehydration mainly by filter-paper and passage into balsam via bergamot oil—and afterwards to tease up a piece of fresh testis in saline and see the mitochondria without any fixing or staining process, transparent indeed and uncoloured, but as clear to a sensitive eye as when stained intensely violet in the permanent preparation.

ii. METHODS REVEALING STRUCTURE

a. Fresh Material

When cells fall apart readily—and unfortunately they usually do not—fresh teased preparations in indifferent media are generally preferable to stained sections. The body fluids of the animal concerned and simple saline solutions (preferably containing some calcium ions⁵) are suitable media. Cytologists would be well advised to concentrate their attention primarily upon cells that can be readily isolated or studied in very small groups, simply because one can thus avoid studying artifacts. Advance is likely to be very rapid now that the phase-contrast microscope has made the examination of unstained living cells so much easier. It is remarkable, however, how much structure can often be made out in unstained cells by patient observation with the ordinary microscope, even when at first glance little is seen.

When no further structure can be discovered in unstained cells, the effect of dyes of small toxicity should be tried. Many basic dyes are suitable, perhaps as suitable as the neutral red and Janus green that have gained such wide acceptance. The dyes may be added to the water in which the animal lives, or injected into it (i.e. into the haemocoel of a mollusc⁵²). Alternatively the dye may be dissolved in a saline solution and the cells suspended in this.

Acid dyes cannot effectively be used supravitaly in this way to show pre-existing structures; but some of them can most profitably be injected into living animals, certain of whose cells will take them up in a characteristic way¹⁴. The dyes which are useful in this respect are chiefly acid disazo dyes, the molecules of which are so large that the solutions may be regarded as colloidal. On account of its low toxicity, high intensity of staining, and resistance to subsequent treatment, trypan blue is probably the best of these. When the cells have had time to take up

the dye, the animal is killed and the tissue cut out. The cells may then in certain cases be examined directly, without further treatment, but it is generally convenient to preserve the tissues in an ordinary fixative and cut paraffin sections, which may be further stained if it is desired. The resistance of the dye to extraction by the solutions in which the tissue is subsequently soaked and in which it is perfectly soluble is very surprising, and must be attributed to the particular physical form in which the enclosing proteins are precipitated by the fixative. Of the dyes other than disazo compounds which may be used intravitaly in this way, isamine blue is the most noteworthy. This acid dye belongs to the triphenylmethane series, and once again has a very large molecule.

The cells of the reticulo-endothelial system have a strong tendency to take up and retain the large molecules of these vital dyes, while failing to absorb or retain, as the case may be, both basic dyes and such acid ones as have small (or extremely large) molecules. These cells are phagocytic, and the taking-up and retention of the vital dyes may be regarded as a process in which they are actively concerned; further, the vacuoles in which the dyes are seen to accumulate may be considered as *sui generis* and not corresponding to any cell element of general occurrence. It is otherwise with the cells of the convoluted tubules of the vertebrate kidney, and here a question of major interest is whether the vacuoles which the dye reveals are to be regarded as having any relation with the vacuolar component of the Golgi element.

The fatal facility of the microtome has made investigators nearly always study cells in sections if it is decided to study them dead. Cytologists will often describe in minutest detail the shape of every structure contained within a cell, while leaving the shape of the cell itself unmentioned or represented by drawings that give no adequate concept. It was not always so. Before the days of microtomy, histologists were expert at the separation of cells by maceration. In all probability few present-day cytologists have tried iodized amniotic fluid as a macerating agent, but in the writer's experience this 76-year-old method⁵¹ is a useful one. Ranvier's remarks⁴⁶ on the techniques of maceration and mechanical dissociation, written in 1875, are well worth study. The early histologists could not only demonstrate relatively gross structure (such as the course of the vertebrate uriniferous tubules) in a way that would be startling in its demonstrativeness to the section-minded student of to-day, but also knew much of intimate cell structure that passes unnoticed when the cell has been through the mill of present-day cytological technique. It is interesting and instructive to turn over the pages of Beale's *How to work with the Microscope*⁹ and to marvel at how much was known before the invention of rocking and rotating microtomes. What is particularly wanted at the present day is a macerating fluid that will preserve the mitochondria and Golgi element in a lifelike form.

b. Fixation

Nowhere in cytological technique has empiricism run riot so freely as in the invention of fixative mixtures. Substances that will obviously react together are mixed haphazard (as e.g. when chromic acid is added to Bouin's fluid¹) and no one can say to what extent the tissues are affected by the original constituents and to what extent by the products of their reaction. Even when no such extra complication exists, the chemistry and physics of fixation are excessively difficult. The speed at which each constituent penetrates, the way in which each acts singly upon each of the different substances present in the cells, how one constituent affects the other two as regards degree of dissociation and chemical action, these are some of the problems that await solution even when so simple and rational a fixative as Bouin's is employed.

The histologist calls a fixative 'good' when the tissues are evenly and slightly shrunken and the nuclei stand out sharply in the stained section, but the sharpness of the precipitated chromatin is no evidence of life-like preservation, and the only evidence of what is good or bad is comparison with the living cell. This point cannot be too strongly stressed: subjective ideas of what final result is good and what bad should never be allowed to form in the mind except on the solid ground of comparison with what is visible in the cell while still unaffected by reagents. Fry²⁴ has insisted that one should not choose for careful study what one thinks to be the 'best-fixed' cells in a preparation, but should pay equal attention to a number of cells chosen at random. The best plan is to observe the action of fixatives on cells of which the structure is known from vital observations. It is reasonable to put some general reliance on fixatives that are known to give lifelike appearances in these cases.

From the welter of different fixative mixtures it is difficult to extract any clear principles for the understanding of why one gives a lifelike preservation while another does not. One fact may, however, be pointed out: most reliable fixatives contain one or more substances which precipitate the proteins of the cytoplasm, and one or more which render those proteins insoluble without precipitating them. This applies to fluids so valuable to the cytologist as Flemming's, Sanfelice's, Helly's, Mann's, and Bouin's. The non-precipitant fixatives in these mixtures are osmium tetroxide, unacidified potassium dichromate, or formaldehyde. One might fancy that non-precipitant fixatives used alone would give the best results, since precipitation causes the formation of artificial structures; but in practice it is found that precipitated proteins are more easily penetrated by the substances used in subsequent treatment, particularly paraffin, and more easily stained. Thus Altmann's fluid (osmium tetroxide and potassium dichromate), excellent in its way, gives an inconveniently crumbly consistency to tissues, and Regaud's (formalde-

hyde and potassium dichromate) fails badly with certain tissues (e.g. the spermatogenetic tissues of many animals). The omission of the non-precipitant fixatives, however, as in Zenker's fluid, results in precipitation of the cytoplasm in a meshwork whose coarseness causes artificial appearances which are serious for the cytologist, though not necessarily for the histologist. (It will be recollected that the potassium dichromate in Zenker's fluid, being in an acid medium, is a precipitant fixative⁶⁰.)

Fixatives containing no protein precipitant are particularly suitable when the freezing microtome is going to be used, because there is then no need to make spaces for an embedding medium.

The mixing of formaldehyde with oxidizers may perhaps be excused on the practical ground that it is done in some very good fixatives, notably in Helly's fluid and those Flemming variants in which formaldehyde is substituted for osmium tetroxide. The writer's fortune in having seen some of Prof. M. J. D. White's beautiful chromosome preparations made with Sanfelice's fluid almost reconciles him to the use of unstable mixtures. The reaction of potassium dichromate or chromic acid with formaldehyde is rather a slow one, and it may be argued in favour of such mixtures that for a considerable time a part of the oxidizer and a part of the reducer are present and exerting their effects on the cells concurrently, the reaction products being of no consequence. This may be so, but it would be worth someone's while to find out just what the reaction products are in the case of a few valuable unstable fixatives (e.g. Sanfelice's⁵⁰), and what effects, if any, they have on cells. Helly seems to have had a feeling that the effects of the reaction products might be damaging, for he recommends that if fixation lasts for more than 6 hours the tissue should be removed from his unstable mixture and transferred to a fluid containing the other constituents but not the formaldehyde.

Until the effects of the reaction products have been investigated, one cannot help being harassed by such thoughts as these. If it is desirable that the dichromate or chromic acid should oxidize something in the cells, why is formaldehyde added, which will interfere with the oxidation, and vice versa, if reduction by formaldehyde is desirable, why add dichromate or chromic acid? Or is one seeking only those effects of these substances that involve neither oxidation nor reduction? The truth is that when one uses these unstable mixtures one knows even less about what one is doing than when a stable mixture is used (which is saying a good deal).

The question whether salts devoid of fixing properties should be added to fixatives to prevent changes due to osmotic pressure is still obscure. For some unexplained reason formaldehyde has long been the fixative to which it is deemed especially desirable to add sodium chloride for this purpose, though it is many years since Meves⁴⁰ treated chromic acid

and mixtures made with it in the same way. It has been shown² that many fixing mixtures are hypertonic, some enormously so, and it is not immediately obvious how the addition of sodium chloride or other salts to them could prevent osmotic changes in the cells. However, Carleton¹⁵ and Young⁵⁸ have found that with certain fixatives and certain tissues there is a demonstrable advantage in adding an indifferent salt. In the absence of the added salt the cells are shrunken. Young supposes that when a piece of tissue is placed in a fixative solution the ingredients of which are slow in diffusion and to which no indifferent salts are added, there is a loss of ions from the intercellular fluids in the unfixed centre of the piece, and hence an osmotic swelling of the cells in the vicinity. He supposes this swelling to go so far as to result in bursting, and he thinks that later the fixative molecules arrive and act upon the already burst—and then shrunken—cells. The last word does not appear to have been said on this subject. The addition of indifferent salts appears to be unnecessary when the fixative contains a considerable amount of acetic acid. When this is not so, it is probably a good general rule to add sufficient sodium chloride (or other suitable salt) to make the osmotic pressure, apart from that of the fixative constituents, slightly less than the osmotic pressure of the body fluids of the animal concerned.

Despite the invention of some useful fixatives in recent years, it remains true that to the present day one of the most valuable is Flemming's fluid (strong formula), first published in 1884²³, which is still among the best both in the intricacies of modern chromosome studies and for the demonstration of mitochondria. For the latter purpose the acetic acid is reduced below 5 per cent., and it is to be remembered that Flemming himself suggested that figure as the maximum to which the acetic acid should rise, and not the standard figure for all purposes.

The whole subject of the chemistry and physics of fixation has recently been treated in considerable detail⁶.

c. Embedding

It is doubtful whether cytologists have made sufficient use of the freezing microtome, which has tended to be a tool rather of the histologist and pathologist. Cells are generally more lifelike in frozen than in paraffin or celloidin sections. In the freezing technique either no embedding at all or mere inclusion in gelatine is required. When once it is familiar the technique becomes remarkably quick and easy.

When embedding in paraffin, it is desirable to pass from absolute alcohol to a mixture of equal volumes of absolute alcohol and toluene (or other dealcoholizing agent). It is probable that no advantage is gained—at any rate with the small pieces of tissue that are used in cytology—by passing from toluene to a solution of paraffin in toluene.

Certainly there is no advantage in passing from a wax of low melting point to one of higher: the wax used in each bath should be the same.

For a reason which is not apparent, the embedding of tissues in paraffin wax affects the capacity to take up dyes. A piece of tissue that will accept carmine readily in bulk may resist the same dye when cut into paraffin sections. Celloidin embedding does not appear to have the same effect.

It has often been assumed that while tissues are embedded in paraffin, they are impermeable to water, but this is far from being true. Material fixed in picric acid often gives a yellow colour to the warm water used for flattening the sections, and conversely it is possible, as Walsem⁵⁶ and others (e.g.³⁹) have shown, to stain sections with aqueous solutions without removing the wax. The method has been used in certain cases in which treatment with alcohol impairs the action of the dye, as in the metachromatic staining of mucin. Paraffin blocks are sometimes soaked in water to make subsequent cutting easier, and it has been thought that the effect of the water is simply to cool the wax. This is not so, for the method does not work unless the tissue is first exposed at the surface of the block. Water by itself, however, is not the most desirable fluid in which to soak paraffin-embedded tissues to soften them, and a special aqueous reagent has been designed for the purpose³, which penetrates paraffin-embedded tissues more readily than water and softens them better. The British Drug Houses Ltd. are marketing a reagent of this kind.

Cytologists usually prefer to embed in paraffin, but celloidin sometimes offers advantages. Very thin sections are not obtainable, but the smaller amount of shrinkage to some extent compensates for this; for the more the cells shrink, the thinner must be the sections if one is to avoid the occlusion of one cell by another. When serial sections are not necessary—and often in cytology they are not—celloidin work takes up little if any more of one's time than paraffin, though the final slide is not obtained so soon. The spaces between cells are probably more faithfully preserved than when paraffin is used, and also it is useful to have a control against the production of artificial appearances by the hot paraffin.

d. Staining

In staining, as in other branches of technique, practice often long precedes theory. When it became known that compounds between methylene blue and eosin presented great advantages in the staining of blood, it was found that the nuclei of parasitic Protozoa in such preparations were stained reddish and not in the same purple colour as the leucocyte nuclei. Since the cytoplasm of the parasites was stained blue, they showed up very distinctly in blood preparations. The complications behind these staining reactions are enormous. The methylene blue

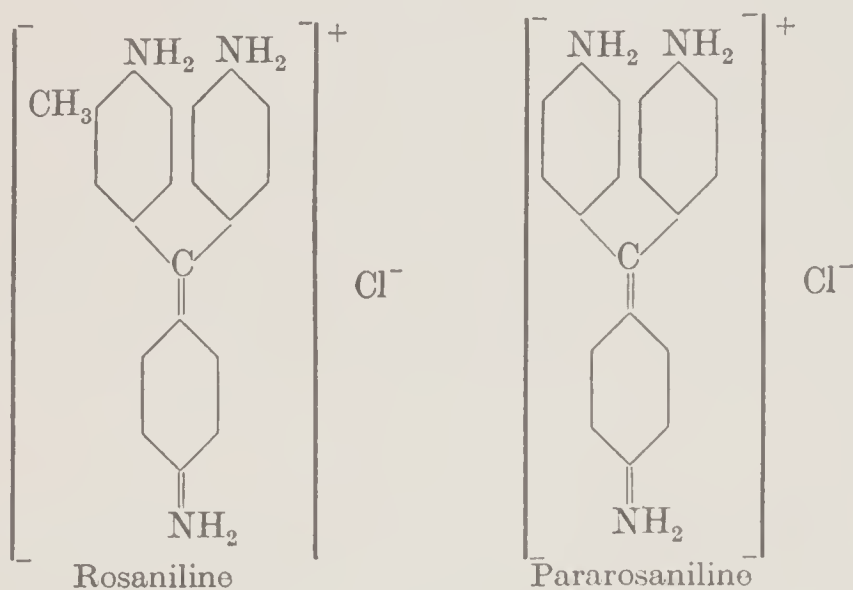
and its derivatives are present in the blood-stains partly as pure substances and partly in combination with eosin. Giemsa showed that methylene blue and eosin alone did not account for the red staining of the Protozoan nucleus, and that it depended on the appearance in the dye of a new substance, not added as such, namely, methylene azure, which has one or two of the methyl groups of methylene blue replaced by hydrogen. He produced the justly celebrated stain which bears his name²⁵ by using a definite quantity of methylene azure as well as methylene blue. It was left to MacNeil to show that even methylene azure is not the direct cause of the desirable red staining of the Protozoan nucleus. Of the two azures, the one with two methyl groups (asymmetric dimethyl thionine) instead of the four in methylene blue, seems to be the more significant in this respect. MacNeil showed³⁸ that the azure acts by transformation to yet another substance, namely, the methylene violet of Bernthsen. He therefore included Bernthsen's violet in his well-known tetra-chrome stain. The complications, however, do not end here, for neither asymmetric dimethyl thionine nor its derivative, Bernthsen's violet, alone stains Protozoan nuclei reddish: a mordant is required. Now resorcinol and a few other substances will act as mordants for this particular staining reaction, and one of the other substances is eosin, itself a resorcinol derivative. Thus the eosin of methylene-blue-eosin blood-stains is in this respect acting as a mordant and not a dye³⁴. As a last complication, the granules of mast cells are stained a reddish colour metachromatically by the methylene blue derivatives, without the intervention of a mordant⁴².

These scarcely credible complications cannot have been dreamt of by Romanowsky⁴⁸ when first he put methylene blue and eosin together in equimolecular proportions and noticed the reddish coloration given to the nucleus of the malarial parasite, never till then differentially stained. In 1901 Leishman produced his simple but excellent stain³⁵, in which the valuable methylene blue derivatives were produced by the action of sodium carbonate on the parent dye. To-day the proportions of the derivatives are generally carefully controlled in blood-stains, but Leishman's product still finds a place in modern technique.

The fact that acidity favours the action of acid dyes and alkalinity that of basic ones has fostered the idea that stains should be dissolved in buffered solutions at definite pH. Levine's careful work³⁶ has shown that pH is not the only factor that matters: different buffer solutions at the same pH, and the same buffer solution at the same pH but different concentrations, have different effects on the intensity of staining. It does not seem important generally to control the pH of staining solutions at all carefully, except perhaps the methylene-blue-eosin blood-stains.

For the study of chromosomes no dye is more used to-day than crystal

violet. (It is probably always better to use crystal violet than that uncertain mixture of violet dyes to which the name gentian is applied.) The method is essentially that introduced into bacteriological technique by Gram. This depends upon the fact that certain basic dyes react in a special way with iodine. While rosaniline and related dyes do not



react with iodine, pararosaniline and other triphenylmethane dyes resembling it in lacking the methyl group (e.g. crystal, gentian, and methyl violets) appear to be capable of forming compounds which may be called iodo-pararosanilines³⁴. These substances have a blue-black colour, different from the violet of the untreated dyes. In Gram's method and its derivatives, the tissue is first stained in the violet dye and then treated with iodine-iodide solution. Certain objects, such as Gram-positive bacteria and chromatin, retard the extraction of the iodo-pararosaniline compound by alcohol and other solvents; others let it go freely. Differentiation in alcohol or in alcohol followed by clove oil produces the desired result.

Hermann in 1889²⁹ took this method from bacteriological technique and used it for staining chromatin in amphibian and mammalian spermatogenesis. He used an aqueous iodine-iodide solution, as did Gram. The modern technique, which has proved so valuable to those working on chromosomes, was introduced by Newton⁴¹, and further particulars have been given by Huskins³⁰ and La Cour³³. The great advantages of the technique are the glassy transparency of the cytoplasm and the even action of the dye over the whole depth of the section, even when it is as much as 40 μ thick. The finest details of chromosome structure are revealed. The rather inexplicable part of the technique is the use of an alcoholic iodine solution and the use of potassium iodide in it. With aqueous solutions the iodide is necessary to dissolve the iodine, but the latter is readily soluble in 80 per cent. alcohol. Just what happens in the very short alcoholic iodine bath is obscure. The alcohol must be tending

to extract the dye as such, while at the same time the iodine must be tending to convert it into the less readily removed iodo-pararosaniline derivative. Whatever actually occurs, nothing remains that shows much further resistance to alcohol, for only a few seconds must be allowed in absolute alcohol before the more leisurely differentiating action of clove oil is brought to bear.

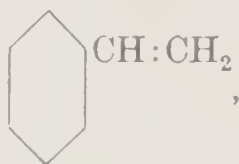
Many valuable dyes are very rapidly removed by alcohol, and it seems worth while to bring to the attention of cytologists an old but apparently almost forgotten method of circumventing this difficulty, much used by Unna and by Pappenheim⁴³. The stained slide is dried by blotting and taken straight into a mixture of alcohol (or alcohol and acetone in equal parts) with 3 times its volume of xylene. During 5 or 10 minutes the alcohol completes the dehydration, while the xylene greatly reduces the speed at which the dye is extracted.

e. Mounting

Glycerine jelly has long been popular for the mounting of frozen sections, but the writer strongly recommends Farrants's medium, which is particularly easy to use. It is sometimes suitable for paraffin sections as well. It consists⁹ of picked gum-arabic, distilled water, and glycerine in the proportions of 2 gm. to 2 c.c. to 1 c.c. A piece of camphor should be added. (See also Kent³¹.)

New synthetic mounting media are now available, some of which may possibly be found superior even to Canada balsam, the virtues of which had seemed to place it almost beyond competition except for special purposes. Certain cycloparaffins are now available which have the necessary properties of ready adhesion to glass on evaporation of the solvent, transparency, lack of colour, neutrality, inertness, permanence, and refractive index (though it would not appear that their optical dispersion has been much studied). Groat²⁷ has found that for the cycloparaffin 'nevillite V', also known as 'clarite', the most suitable solvent is toluene. A solution at 60 per cent. gives a convenient consistency, and the material is used exactly as though it were a solution of balsam in xylene. The refractive index at the D-line only differs by 0.009 from that of balsam, and the neutrality and absence of colouring with age are definite advantages. It remains to be seen whether experience will expose any defect in this promising medium.

The styrene derivative 'distrene 80' has been recommended by Kirkpatrick and Lendrum³², who have studied the suitability as mounting media of a number of natural resins and synthetic substitutes. Styrene itself,



is a liquid which may be obtained from the plant product storax or synthesized in the laboratory. It polymerizes on heating to a glassy substance. The exact chemical nature of distrene 80 does not appear to have been disclosed by the manufacturers (Messrs. Honeywill and Stein Ltd.), but it is stated to have the enormous molecular weight of about 80,000. It is readily soluble in xylene and the solution is colourless. A simple solution is not desirable, however, on account of the tendency for the medium gradually to shrink back under the cover-slip. A plasticizer, tricresylphosphate (a colourless liquid, or solid if perfectly pure), serves to prevent, or at least reduce, the tendency of distrene 80 to retract under the cover-slip of microscopical preparations. Kirkpatrick and Lendrum have shown that their plasticized distrene does not tend to change in pH on keeping, and the writer confirms their finding that even the blood dyes, which are very sensitive to changes in pH, retain their colours when this medium is used.

f. Demonstration of Mitochondria and the Golgi Apparatus

Whenever possible, mitochondria should be examined in living cells, where they can usually be seen with the ordinary microscope without staining. Phase-contrast microscopy makes them particularly evident. When they are to be vitally stained, a reliable specimen of Janus green must be obtained. It is desirable to have a standard test-object (such as the primary spermatocyte of the snail) on which to test any new specimen of the dye. Janus black sometimes gives good results when the green does not.

When mitochondria are studied in sections, some variant of Altmann's technique is generally used: that is to say, they are stained by a hot concentrated solution of acid fuchsin in aniline water. Several ways of differentiating this dye have been proposed; most of these are difficult to carry out. The simplest method is that of Cain¹³, who uses a very weak solution of sodium carbonate. (The advantage of using a colourless differentiator is considerable.) Cain then stains the nuclei with water-soluble methyl blue, an acid dye having considerable affinity for chromatin. A great advantage of this method is the fact that there is no hurry at any stage, and the results are excellent. It is doubtful whether trichrome methods have any real advantage over this, and they are much more difficult to carry out successfully.

The time has now gone by when it was profitable simply to apply the standard 'Golgi methods' and record the results. The study of the Golgi element has now reached a turning-point. Da Fano²⁰ himself admitted that his well-known method sometimes darkens the mitochondria. Thomas's recent studies^{52,53} make it probable that the black objects seen after the familiar techniques are in some places spheroids (the neutral red vacuoles of Parat⁴⁴, with their lipoidal sheaths⁵), in other

places mitochondria, and in others again merely artificial deposits of reduced osmium or silver, not representing anything present during life. The name 'Golgi apparatus' is thus equivocal. In many cases, however, the mitochondria are not blackened and the reduced osmium or silver is confined to a definite part of the cell. When this part is examined during life, it is found to contain either spheroids or small particles of material resembling in its reactions the lipoidal sheaths of spheroids. These, then, seem to be the objects to which the name 'Golgi apparatus' should refer, if it is found desirable to retain this terminology.

No one should undertake research in this field without first studying the papers of Worley and Worley⁵⁷ and of Thomas^{52,53}, who have developed original methods of attack. The present writer has also introduced some new techniques⁵. Using methylene blue as a vital stain, the Worleys have been able to watch the Golgi apparatus actually performing its synthetic functions in the living cells of embryonic and larval molluscs. Thomas has studied living neurones of *Helix* and mammals and devised methods of retaining the Golgi apparatus in its natural form in fixed preparations. He has also studied steps in the production of the familiar artefacts.

Cytologists have been misled by the current idea that the Golgi element is invisible during life. It is, on the contrary, often one of the most visible objects in the living cell, easily seen without any staining or other treatment whatever. Difficulty arises simply from the fact that people are looking for something resembling the osmium or silver artefact, and they find nothing of the sort—because it is not there.

iii. METHODS REVEALING CHEMICAL COMPOSITION

a. Introduction

If sections of tissues are stained lightly with xyloidine red, treated with phosphomolybdic acid, and then placed in a solution of light green, the latter dye will soon begin to colour the white fibres of connective tissue. Parts of the section can be recognized by morphological characters to consist of connective tissue, and one can easily arrange to stop staining when these are bright green while the background is pink or red. This method is very valuable in studies of the morphological distribution of white connective tissue fibres. It has been chosen as a typical example of a colour-reaction that does *not* constitute a histochemical test. One decides on purely morphological grounds when to stop staining with light green; if the dye were allowed to act for a longer period, everything in the section would eventually give the green reaction; and if one stops staining when the white fibres are green but the background still pink, mucin will also be green. No chemist would think of using light green as a reagent for collagen. The fact that light green has an affinity

for white fibres is of course dependent on the chemical composition of the fibres, but in the present state of knowledge the dye cannot be used in chemical identifications.

Histochemistry is *the microscopical study of the location of chemically-recognizable substances or groups of substances in the tissues of plants and animals*. It thus includes the study of the metabolic changes in tissues, in so far as the sites of these changes can be located by the microscope; but dynamic histochemistry is largely a subject of the future. Cytochemistry is nearly synonymous with histochemistry, because histochemical methods seldom give uniform reactions throughout the substance of a cell and observations are therefore necessarily carried out at the cytological level. The study of the chemical composition of inter-cellular matter, however, belongs to histochemistry and not to cytochemistry, and the former is thus the larger science, which includes the latter. Microchemistry is the study of the chemical reactions of very small quantities of materials, not necessarily with any relation to biology. The word is sometimes used loosely as a synonym for histochemistry.

The status of histochemistry at the present day can only be understood by a history of the science. The present writer has elsewhere⁴ given a first sketch of such a history. The founder of the science was Raspail, who began in 1825 by introducing the iodine test for starch into microscopy⁴⁷, and thereafter devised many valuable histochemical methods. The early minute anatomists from Raspail's time onwards were histochemists as a matter of course. Henle, for instance, quite naturally devotes a considerable part of his *Allgemeine Anatomie* (1841)²⁸ to observations on chemical composition. The science of histochemistry was struck a severe blow, however, by the repeated rediscovery during the years 1848–58 of the use of dyes in microscopical technique. Dyes gave a huge impetus to purely morphological studies, and the great majority of microscopists in the second half of the nineteenth and the beginning of the twentieth century were morphologists who lacked a chemical outlook. It was found easy to stain one tissue-constituent in this colour and another in that, and people without chemical knowledge began to build up a sort of parachemistry of the tissues—an array of weird names not related in any way to the general body of chemistry itself. Substances were labelled pyrenine, linin, plastin, &c., without any serious attempt being made to identify the substances so named in terms of real chemistry. When a new cell-inclusion turned up, which stained in one of the characteristic ways, it was labelled with the corresponding name, and no one took any trouble to find out what was its actual chemical composition.

This outlook is not dead to-day, but a change in the right direction has occurred. The true spirit of histochemistry was never completely

extinguished by the dyers, and good work continued to be done, especially in the botanical field. In 1910 Prenant, in a valuable paper⁴⁵, collected together the real knowledge of the subject available in his day. The turning-point came, however, with the publication of Lison's *Histochimie animale* in 1936³⁷. A standard of chemical validity was set by this work which raised the science to a new plane. The influence was of course felt particularly in the field of animal histology and cytology, partly because the book was addressed primarily to workers on animal tissues and partly because botanists had throughout maintained much higher standards in this subject.

b. A Proposed Classification of Histochemical Methods

The time seems appropriate for the development of a logical classification of histochemical methods, and this will be attempted here. In the past, writers have been content to group the available methods together in an arbitrary way that could not form the basis of a logical classification. Histochemists should carefully consider what is supposed to be the source of the validity of every test they use; and the writer believes that clear thought on this subject demands a rational grouping of methods.

If a general review be made of histochemical methods as a whole, it will be found that they fall naturally into three groups. In the first, the object under investigation reveals its chemical composition while remaining in the tissues (e.g. by a colour-reaction). These are the '*non-destructive in situ methods*'. In the second group the object reveals its composition by being destroyed *in situ* (e.g. by being dissolved by a specific enzyme). These are the '*destructive in situ methods*'. In the third group the object is separated from the tissues (e.g. by centrifuging) before analysis, the microscope being used to make sure that the object removed is the same as that previously located in the tissues. These are '*extra situm methods*'.

The first two groups of methods are subdivided on the basis of those characters possessed by the object under investigation that reveal its chemical nature. The subdivision of the third group cannot logically proceed on this basis, because the fundamental differences between one method and another depend on the methods of extraction adopted. When once the substance has been extracted and microscopical control has shown that it is a histologically-localized tissue-constituent, analysis may proceed according to the standard methods of analysis. There would obviously be no point in classifying these methods here. The absorption spectrum may be examined, or ordinary chemical analysis used. Since the amount of substance obtained is usually small, microchemical methods find application here.

The proposed classification is as follows:

- I. NON-DESTRUCTIVE IN SITU METHODS; analysis is effected by determination of the object's:
 - i. *Refractive index.*
 - ii. *Optical sign* (positive or negative) in polarized light, and *degree of birefringence.*
 - iii. *Number and inclination of optical axes*, as revealed by convergent polarized light and other methods.
 - iv. *Geometrical crystallographic properties.*
 - v. *Capacity to absorb electro-magnetic rays of certain wave-lengths*, as shown by
 - A. colour;
 - B. the absorption spectrum produced when a wide range of wave-lengths is used simultaneously;
 - C. the absorption of rays of a single wave-length.
 - vi. *Capacity to fluoresce when illuminated by ultra-violet light* ('primary fluorescence').
 - vii. *Resistance to solution.*
 - A. without previous chemical alteration:
 - a. by ordinary solvents;
 - b. by a saturated solution of the suspected substance;
 - c. by enzymes.
 - B. after previous chemical alteration.
 - viii. *Capacity to dissolve certain colouring agents.*
 - ix. *Capacity to adsorb certain dyes selectively.*
 - x. *Capacity to dissolve or adsorb certain substances that will fluoresce in particular colours when illuminated by ultra-violet light* ('secondary fluorescence').
 - xi. *Capacity to interact chemically with reagents, with production or change of colour.*
 - xii. *Resistance to micro-incineration.*
 - xiii. *Reaction as an enzyme with a provided substrate, the reaction product being:*
 - A. itself coloured;
 - B. capable of demonstration by colouring agents.
 - xiv. *Radioactive properties* (after administration of radioactive isotopes to the organism).
- II. DESTRUCTIVE IN SITU METHODS; analysis is effected by determination of the object's:
 - i. *Melting-point.*
 - ii. *Solubility:*
 - A. in solvents;
 - B. by enzymes.

- iii. *Emission spectrum.*
- iv. *Capacity to give rise to recognizable crystals when treated in particular ways.*

III. EXTRA SITUM METHODS ; separation is effected by :

- i. *Direct mechanical means* (e.g. teasing) ;
- ii. *Solution* (especially applicable to pigments) ;
- iii. *Differential centrifuging* ;
- iv. *Micro-sublimation.*

Some of the methods, especially those falling in groups I. iii and II. iii, stand as yet only on the borderline of histochemistry, because the substances identified cannot generally be exactly located with sufficient accuracy to justify the use of the compound microscope.

c. *The Validity of Histochemical Tests*

It is of course often desirable to check the results of one method by the use of others. It is right to be suspicious even of many generally accepted methods. Danielli has published a useful warning on this subject²², though it may be argued that he carries caution to excess. Methods relying on resistance to solubility (I. vii) are particularly liable to error, because substances in tissues may have their solubilities reduced by association with other substances (e.g. they may be adsorbed on insoluble proteins).

Many methods involving capacity to adsorb certain colouring agents selectively (I. ix) and to interact chemically with production or alteration of colour (I. xi) are based on purely empirical knowledge, without the chemical or other reactions involved being understood. Such methods are only reliable when there is inductive evidence that among all the substances occurring in organisms only one substance, or one group of substances, reacts positively. A really comprehensive study of this kind has not very often been made. The writer has recently published^{7,8} a method for the histochemical recognition of phospholipines, depending for its validity on exclusion-experiments of this kind. When a test does not depend on some evidently valid basis (for example, an absorption or emission spectrum, or a recognized chemical reaction for which one can write the equation), no one should be content to use it without finding exactly what is supposed to be the basis of its validity; that is, what proof there is that other substances or groups of substances cannot react positively to it.

Care must be taken to find whether a colour-reaction at a certain place in a cell genuinely indicates that the substance that gave the reaction was present at precisely that place, or whether, on the contrary, the visible reaction-product may have moved from one part of a cell to another²².

Although the writer's main purpose is to insist on the necessity for high standards of validity in histochemistry, yet there is still a place for certain carefully chosen rough-and-ready guides. The histochemist will do well to provide himself with some quick and simple methods that can act as pointers. Zacharias's method for proteins might be cited as an example⁵⁹. Pyronine and methyl green (Unna⁵⁴) is another: whatever is coloured red by the use of this mixture is quite likely to contain ribosenucleic acid. Such methods may greatly reduce the number of critical tests that must be applied to establish the chemical identity of tissue constituents. They must be used, not as evidence of chemical composition, but simply to suggest what valid tests should be tried first.

d. Modern Tendencies

Methods falling within the following groups of the above classification are of particular interest at the present time:

- I. v. C (e.g. Caspersson's method¹⁶ for the nitrogenous bases of nucleic acids).
- I. xiii. B (e.g. Gomori's method²⁶ for phosphatase).
- II. ii. B (e.g. Brachet's method^{11,12} for ribosenucleic acid).
- III. iii (e.g. Bensley and Hoerr's method¹⁰ for isolating mitochondria).

One of the chief needs in modern histochemistry is the development of quantitative *in situ* methods.

The discovery that certain enzymes are resistant to paraffin embedding and can reveal themselves by their activity in sections is an important one, well exploited by Gomori²⁶. For a critical study of Gomori's method, see Danielli²¹. In developing new methods of this kind, the difficulty will be to make the products of enzyme activity visible at the exact sites of their origin.

The discovery of methods for preparing pure enzymes is likely to revive an old principle of histochemical technique. Unna⁵⁵ used the idea of ascertaining the staining reactions of various tissue components, applying solvents to sections, and then using the appropriate stains to find out whether these components had been dissolved out. This method of 'Chromolyse' (a subsection of II. ii. A) broke down because substances occur in tissues in complex mixtures, in which one substance affects the solubility of another. Pure enzymes are superior to solvents in this respect, and Brachet^{11,12} has introduced what is essentially chromolysis by enzymes (see also Sanders⁴⁹). Ribosenucleic acid has a particular affinity for pyronine, which cannot, of course, be used as a histochemical test since it is simply a basic dye with no actual specificity. If, however, an object in a cell is readily stainable by pyronine in an ordinary section, but unstainable after a section has been treated with

pure ribonuclease (which removes ribosenucleic acid from histological sections), it is legitimate to conclude that the object contains or consists of ribosenucleic acid. This kind of test is likely to come into common use.

The methods in groups I and II are generally reliable as regards location of the object in tissues, but often unreliable as regards chemical composition. The group III methods, on the contrary, can be made reliable as regards chemical composition but are often unreliable as regards location (i.e. site of origin of the substance extracted and analysed). An example is provided by the material that Claude^{18,19} isolates from cells by differential centrifuging and describes as 'microsomes'. It has been questioned whether this material actually occurs in living cells, as he claims, in the form of minute granules. However this may be, there is very strong reason to believe that mitochondria can be isolated by centrifuging: the weight of evidence seems opposed to Claude's view¹⁷ that Bensley and Hoerr's granules are not actually mitochondria. Claude has himself¹⁸ isolated from other cells and analysed what seems undoubtedly to be mitochondrial material. The earliest analyses of mitochondria obtained by centrifuging gave too low a figure for phospholipine, but later figures obtained by various authors are reliable. Bensley and Hoerr's method¹⁰ for isolating very minute cellular constituents is a landmark in the progress of histochemical technique.

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CHAPTER I (*cont.*)

CYTOLOGICAL TECHNIQUES

B. SPECIAL METHODS

By F. K. SANDERS

i. INTRODUCTION

WORK on the structure and organization of the cell during recent years has received a great impetus from the introduction of several new types of procedure. These have been: (1) the 'frozen-dehydration' method of tissue fixation, whereby tissues may be dehydrated and embedded without the changes which normally follow the use of chemical fixatives and dehydration media; (2) methods for the fractionation of cells by physical methods, so that portions of their structure may be collected and analysed in bulk; (3) special histological methods for the localization of sites of enzyme activity in tissues; and (4) the use of enzymes as histochemical reagents. The science of micro-dissection has been helped by the introduction of new and more effective instruments.

Since the increased use of such methods may well have a great influence on the direction taken by cytology in the next few years, it has been thought profitable to give some account of them here. It has not been our intention in this chapter to review exhaustively the advances made with these methods, and for detailed descriptions of the various techniques the reader is referred to the original papers. In the present account the general scope of the various methods will be indicated, and some account given of points at which they may possibly be open to criticism.

ii. THE FROZEN-DEHYDRATION METHOD OF TISSUE PREPARATION

a. Basic Principles

The treatments involved in the making of histological preparations normally bring about many alterations in the tissues subjected to them. Not only are proteins greatly altered in solubility, but mineral constituents, fats, and the like are frequently moved from their original location in the tissues. These unfavourable aspects of histological technique have long been the bane of the cytologist, and in an attempt to overcome them the frozen-dehydration method has been introduced. This changes the tissues little, if at all, apart from the removal of water. The basic principle of the method is as follows: tissues are frozen as rapidly as possible; then they are placed in a high vacuum in a low

temperature, where the ice contained in them evaporates without melting, thus preventing the shifting of materials which would follow the removal of water by other means. Following this freehand sections may be cut, or the tissue embedded in wax without further ado.

Frozen-dehydration was first used by Altmann¹. It was later revived by Gersh⁴⁰, and many of the earlier difficulties of the method have been overcome by refinements introduced by Hoerr⁵², Scott⁸⁰, Simpson⁸², and others⁴⁷. The chief drawback of the method is that it requires the use of special apparatus, which is often expensive. Many of the early types of equipment were, however, needlessly elaborate, and it is now possible to construct equipment which is within the means of any moderately equipped laboratory. The two chief problems of the method are to achieve sufficiently rapid freezing, and to secure rapid drying at sufficiently low temperatures.

b. Freezing

Tissue frozen too slowly is badly distorted by the formation of large ice crystals, which give it a vacuolated appearance, cells actually being ruptured by the growth of ice crystals within them. Gersh⁴⁰ employed liquid air in order to overcome the distortions occasioned by slow freezing. Unfortunately, liquid air boils violently when tissues at room temperature are thrown into it, and they become surrounded by a jacket of vapour which retards heat transfer. This results, in the case of large blocks, in slow cooling of the interior, with the consequent formation of large ice crystals. This defect has been made less troublesome by immersion of the tissues to be frozen in liquids such as isopentane, chilled to -170° C. with liquid nitrogen⁵²; the latter is used in preference to liquid air on account of the ease with which isopentane forms an explosive mixture with liquid air. The isopentane is agitated during tissue immersion to ensure rapid heat extraction. Even with isopentane a sort of supercooling can occur in parts of a tissue block, and some ice-crystal formation takes place. The patterns, however, are geometrical and easily detectable microscopically. Consequently, the tissues which show the best preservation by this method are those with a low water and lipid content. Tissues such as testes and nerves usually show some ice-crystal artefact.

With isopentane it appears that the main factor limiting the rate of heat transfer from the tissue to the medium is the heat conductivity of the instantaneously frozen surface of the tissue⁸²; this is a factor which it is not possible to control. It has been found that the organs least distorted by the freezing process are those with a loose lobular structure, like the pancreas. The considerable connective tissue portions of these organs probably freeze first by propagation of ice crystals inwards. The ice formed in this way is a better conductor of heat than

the still fluid interior, and allows a rapid withdrawal of heat from the cellular portion which it encloses.

Apart from beakers and vacuum flasks to hold the necessary liquids, no special apparatus is necessary at this stage of the process.

c. Dehydration

In order to avoid melting, with its consequent vitiation of the purposes of the technique, dehydration must be carried out at a temperature below the eutectic point of the tissue. There is, unfortunately, at present no way of knowing what the eutectic point of so complex a mixture of substances as living tissue may be. However, there are reasons for believing that the absolute depression of freezing-point in tissues may be very low, indeed below -50°C . At any rate, a more lifelike appearance is obtained by dehydrating tissues below -30°C . An apparatus for dehydrating frozen tissues must thus include some means for maintaining the tissue at a temperature below -30°C . during the whole process, which may take several days. In this respect the process of dehydrating tissues differs from the lyophile process used for dehydrating sera and other biological fluids. In the latter process³⁶ the material is shell-frozen in small quantities in separate ampoules and thus presents a very large evaporating surface. Under moderate vacuum water evaporates sufficiently fast for the material to be kept frozen in supplying the latent heat of evaporation of the ice. In the case of tissues the bulk/surface ratio is much greater, and some form of artificial cooling must be used. In the early types of equipment this was a mechanical refrigeration system⁴⁰. This was elaborate and has since been replaced by simpler systems. Taylor⁸⁸ has described an apparatus in which the dehydration chamber is kept cold by the circulation of cold carbon dioxide, the system operating according to the principle of a domestic water-heating plant; an ingenious thermostatic device regulates the temperature of the chamber by controlling the rate of CO_2 flow. A similar arrangement has been used with success by the author. In the excellent cryostat of Packer and Scott⁷³ cooling is achieved by surrounding the drying chamber with an annular space in which the air pressure is reduced; the whole chamber with its jacket is further surrounded by a Dewar flask full of dry ice. With a pressure in the jacket of 1 mm. Hg. the temperature at the surface of the tissue is -65 to -70°C .

At these temperatures the vapour pressure of ice is extremely low (0.002–0.003 mm./Hg.), and a very efficient high vacuum system is therefore necessary to ensure evaporation from the tissue. A two-stage mercury or oil diffusion pump with a pumping speed of at least 10 litres per sec., backed by a rotary pump of the Hyvac type, is necessary. Some absorbent is also necessary for the water vapour evaporated dur-

ing dehydration. This can be a tray of phosphorus pentoxide, inserted in the line as close to the tissue as possible. In the Packer and Scott cryostat contamination of the pump oil is further guarded against by a liquid air trap between the diffusion pump and the dehydration chamber. A further trap between the diffusion and backing pumps is necessary to prevent contamination of the latter with oil or mercury from the diffusion pump.

The time required for dehydration varies both with the size of block and the type of tissue used, and a standard time cannot be laid down. While water is coming away from the tissue, however, there is a pressure gradient between the dehydration chamber and the diffusion pump. When evaporation ceases this gradient disappears. It can therefore be used as a measure of the dehydration of the tissue. This can be done by means of a pair of ionization gauges, one placed as close to the tissue and one as close to the pump as possible; alternatively, a pair of Pirani gauges may be used, and the pressure in the dehydration chamber compared with that in a standard evacuated bulb. Evaporation may be judged to have ceased when the pressure in the chamber, measured by either of these types of apparatus, declines to a low and constant level. It is customary to continue pumping for a day or so after this level has been achieved, as certain tissues appear to give off their water in a series of bursts.

At low pressures the speed of gas flow through a tube varies with the third power of its radius, and inversely with its length. In designing apparatus for dehydration it is therefore important to keep the connexion between the dehydration chamber and the pumping system as short and as wide-bored as possible.

d. Applications

In ordinary histology frozen-dehydration does not possess any marked advantage over classical methods of fixation, except that tissues so preserved may resemble more closely their living structure. However, the method has immense advantages in the study of materials in tissues which are destroyed or moved by the normal processes of histological preparation; it has thus been used with conspicuous success in the preparation of tissues for the investigation of mineral distribution⁸¹. The method is also well adapted for the histological study of rapid physiological changes in tissue. An extremely ingenious use of the method in this respect is the study⁴² by Gersh and his collaborators of the formation of gas bubbles in tissue during decompression. By far the widest field for the use of the method, however, is likely to be that of histochemistry. Using frozen-dehydration it should be possible to devise techniques for the histochemical localization of many enzymes which are normally destroyed by the process of fixation and dehydration.

Moreover, frozen-dehydrated tissues should be useful for the histological control of biochemical extraction procedures, and have already been used in this connexion by Bensley and Hoerr⁵. Further possible uses are the preparation of tissues for fluorescence microscopy, and for storage with a view to their later surgical use⁹¹.

iii. CENTRIFUGAL FRACTIONATION METHODS

a. Basic Principles

The basic principle of such methods is the maceration of tissues (by mincing, grinding, squeezing through silk or towelling, or trituration in a high-speed mixer such as the Waring blender), and the separation of the resultant mush into fractions of different density by successive cycles of centrifugation at different speeds. All operations are carried out as close to 0° C. as possible, if practicable in a cold room. By such means it has been claimed that it is possible to obtain fractions consisting largely of the following cellular constituents: nuclei^{8,32,33,67,85,92}, chromatin strands²², chromosomes⁶⁸, zymogen granules¹⁷, melanin granules¹⁶, chloroplasts⁶⁶, particulate glycogen⁵⁹, mitochondria^{5,20,54,55,60,79}, and sub-microscopic particulates termed microsomes^{9,10,15,18,20,84}. In certain cases these general methods have been supplemented by enzymatic digestion to get rid of unwanted protein⁸⁵.

Chemical analyses and determinations of enzyme content have been made on the fractions so separated, and the content and activities observed referred to cell structures demonstrable by classical cytological methods. However, except in certain well-defined cases, little evidence has been offered that such fractions are in fact identical with known cellular structures. Moreover, it cannot be assumed that because a certain enzyme is found in isolated 'nuclei' or 'mitochondria' it is, in fact, present in this location in the living cell. Cells are highly complex colloidal systems, in which the distribution of substances between the different parts of the system is likely to be altered by procedures far milder than those listed above. Such criticisms do not invalidate isolation methods of this type, but the onus is upon the worker who claims to have isolated a specific cellular component and demonstrated its chemical composition and enzyme activity to show (*a*) that the particle he has so isolated exists as such in the cells of the tissue used, and (*b*) that the activities he demonstrates are, in fact, localized at these sites in cells. With these reservations, fractionation methods are likely to be, and have already proved, very valuable tools in cytological work.

b. Nuclei

Chambers¹³ and others have shown by micro-dissection methods that a nucleus removed from a cell into a foreign environment either dissolves or sets to a rubbery gel. Hoerr⁵³ points out that the nuclei of guinea-

pig liver readily fragment in water at the neutral point, or in salt solutions more hypotonic than 0.50 per cent. However, fragmentation of cells in weakly acid solutions of a pH around 6.0 seems to render the nuclear membranes tough and rubbery, so that adequate preparations of nuclei may be obtained from homogenates prepared by treatments varying in severity from mild grinding to violent beating in a Waring blender. Consequently, many workers have succeeded in isolating 'nuclei' in bulk from macerated tissue: citric acid solutions varying from 0.004 to 0.26 M have been used^{32,33,67,85,92} as maceration media, and nuclei have later been isolated by filtration through silk or cheese-cloth followed by differential centrifugation, the required fraction sedimenting at 1,000–1,500 g.

There is little doubt that the bodies seen in such preparations are derived from cell nuclei. The latter can be seen microscopically in the process of ejection from cells when the latter are treated with 5 per cent. citric acid²³. It cannot be denied, however, that such isolated nuclei are probably much altered from their living condition, and may have gained or lost substances to the rest of the cell debris. There is no reason at present to accept inferences that enzymes such as arginase, found by some workers³³ to be associated with isolated nuclei, exist in or on the nucleus when in its cellular environment. So far as I am aware, no tests have been made of the adsorptive capacities of isolated nuclei, nor has there been any investigation of possible variations in their chemical content when isolated under different conditions. Until this is done we can have no idea as to possible shifts of substances which may occur when the cell is disrupted. Although the high content of isolated nuclei in alkaline phosphatase can be confirmed on whole cells histochemically⁹, even the latter evidence is not beyond criticism^{64a}.

c. Mitochondria

A fraction containing particles 0.5 to 2.0 μ in diameter can be obtained from certain tissues (liver, heart, &c.) by freeing the suspension of nuclei, whole cells, and debris by a preliminary slow centrifugation, and then spinning down the required particles at about 2,000 g; the resulting sediment is then freed of extraneous material by further alternating cycles of centrifugation. Such a fraction was first noted by Warburg⁹⁰ who satisfied himself that the particles obtained corresponded to granules which he could see leaving crushed cells in the suspension. Since then cellular particulates of this size range have been prepared by many workers, notably Bensley and Hoerr⁵, Claude^{18,20}, Hoerr⁵³, Hogeboom *et al.*^{54,55}, and Schneider⁷⁹. Considerable differences of opinion exist as to the best maceration medium. Claude¹⁸ at first used water to which a few drops of NaOH had been added to make a pH of about 9.0. In later studies²⁰ he used 0.85 per cent. NaCl buffered at pH 9.0 to 9.5.

The workers of the Bensley school⁶⁰, however, insist that while the use of alkaline media prevents aggregation of the particles, it also hastens their disintegration; they recommend saline buffered at pH 6.3. Hogeboom *et al.*⁵⁵ use 0.88 M sucrose, on the grounds that it allows particulates to be isolated which correspond most closely to mitochondria; owing, presumably, to the considerable viscosity of the sucrose solution, a field of 24,000 g was used to sediment the particles.

The cytological identification of the components making up this fraction is an important and difficult problem. Bensley and Hoerr⁵ originally identified it as mitochondria on evidence that it contained granules staining with the Altmann stain. Claude²⁰ more cautiously terms this the 'large granule' fraction in contrast to the 'small granules' or microsomes obtained at still higher centrifugal speeds (see below). Hogeboom *et al.*⁵⁵ again identify the fraction with mitochondria on the basis of staining reaction. The size, shape, and staining reactions of the components of the 'large granule' fraction of mammalian liver when isolated in different media are given in the accompanying table:

Medium	pH	Size	Shape	Staining reactions	
				Janus green	Altmann
Water	9.0	1-5 μ	Spheres	—	—
0.85% saline	7.3-9.5	0.5-2 μ	Spheres	—	+
0.85% saline	6.3	1-2 μ	Spheres + Rods	—	+
0.88 M sucrose	—	0.2-1 $\mu \times$ 1-5 μ	Rods	+	+

Fractions prepared in saline contain numbers of spherical bodies of the order of size of mitochondria, which react to the Altmann stain, but not with Janus green; water homogenates contain much larger, paler, non-staining spheres. The fraction prepared in sucrose, however, contains particles which are rod-shaped and able to accumulate Janus green from very dilute solutions of the dye, both properties specific to mitochondria within the living cell. Although not conclusive, as no evidence based solely on a staining reaction can be, it appears likely that the granules prepared in this way do really correspond to mitochondria, and this is the method of choice for their preparation at the present time⁵⁵. It must be emphasized, however, that the centrifuge does not select mitochondria as such, but merely particles of a certain density. 'Mitochondrial' fractions, therefore, may easily be contaminated with material of different origin but similar density. Indeed, Claude²¹ considered his 'large granule' fraction from mammalian liver to consist of

a mixture of mitochondria and 'secretory granules' in unknown proportions depending on the physiological state of the liver extracted.

In view of the ease with which the size and shape of 'large granules' may be changed by varying the solute concentration and pH of the maceration medium, it would appear probable that reports of their chemical composition or enzyme content could have but little bearing on the composition of mitochondria in the living cell; the same objections put forward in the case of nuclei would apply here with even greater force. Moreover, Claude²¹ has shown that strongly acid and alkaline solutions extract pentose nucleic acid and nucleotides from such granules, and that in distilled water they disintegrate with the liberation, among other substances, of a yellow pigment and bodies resembling microsomes. It is therefore surprising that authors using very different methods of extraction agree in ascribing succinoxidase and cytochrome oxidase activity to these granules^{27,54,60,79}. Indeed, in 0.88 M sucrose extracted liver the extracted granules have 70 to 80 per cent. of the succinoxidase activity of the original extract⁵⁴. Such persistently high recovery of the enzyme in the large granule fraction after repeated sedimentation makes it doubtful that this finding is simply the result of adsorption, particularly in view of the fact that succinoxidase is a complex system of several components. Reports^{19,79} of the segregation of other enzymes such as adenylypyrophosphatase and phosphatase in this fraction, however, must be treated with reserve, in view of the finding⁵¹ that intracytoplasmic particles such as virus elementary bodies are able to adsorb phosphatase, catalase, and lipase from solutions containing these enzymes.

d. Microsomes

Following the removal of nuclei and 'large granules', subjection of saline²⁷ or phosphate¹⁰ tissue extracts to still more powerful centrifugal fields (18,000 g) results in the separation of a second particulate component, composed of elements 50 to 300 m μ in diameter, termed 'microsomes' by Claude¹⁵. They were studied extensively by Claude^{18,20,&c.} and Brachet⁹ and others, and have been obtained from many tissues and species¹⁸. Considerable interest attaches to these particles on account of their physical resemblance to infective particles isolated from the Rous sarcoma of chickens¹⁴.

Some evidence exists for the pre-existence of particles resembling microsomes in the living cell. Claude^{18,21} subjected *Amphiuma* liver in cold saline to a centrifugal force of 18,000 g, and examined frozen sections of the tissue after centrifugation: the cells became stratified into four zones; the lowest (unstained) consisted of particulate glycogen; the next contained the nucleus, mitochondria, and 'secretion granules'; the next was uniformly basophil, and assumed to consist of microsomes since the

latter show basiphilia *in vitro*, and have been shown chemically to contain much pentose nucleoprotein; the uppermost zone consisted of hyaline cytoplasm. A similar result was obtained by Brachet and Jeener¹⁰ on frog liver. Sections of liver centrifuged at 40,000 g in cold sucrose gave a positive pentose reaction localized in a lunular zone close to the nucleus. Moreover, dark-ground examination of living cells under very intense illumination revealed⁴ that protoplasm, which appeared clear under transmitted light, contained an immense number of sub-microscopic particles in active Brownian movement. While this evidence is not conclusive for the identity of microsomes with pre-existing particles in the living cell, it does show that particles of this order of size are at any rate present in cells.

Isolated microsomes contain pentose nucleic acid, protein, and up to 50 per cent. of their dry weight of lipide, according to analyses given by various authors^{10,18,21}. The composition of microsomes isolated from widely differing tissues is remarkably uniform^{15,17}. Reports as to the enzyme content of microsomes are conflicting. Claude²¹ states that they are largely without enzyme activity; Brachet and Jeener¹⁰ find a number of enzymes associated with this fraction, including respiratory enzymes. Part of this discrepancy can be explained, no doubt, on the basis of contamination of the microsome fraction by large granule material; alternatively, the enzymatic activity of the large granule fraction may be due to contamination with microsomes. Until conditions of separation are better standardized it will not be possible to apportion activities between these two fractions. In addition, there is evidence that microsomes are able to adsorb material from extracts in which they are set free. The microsome pellet made from extracts of unperfused livers is cherry red owing to adsorbed haemoglobin, whereas that from perfused livers is not so coloured¹⁸. As in the case of the other components of cell extracts, much further work will be needed before we have any idea of the enzymes associated with such minute particles in the living cell.

e. Chromosomes

Claude and Potter²² were the first to isolate 'chromatin' threads from lymphoid tumours and from liver by combining the action of a high-speed mixer in breaking nuclei with subsequent differential centrifugation. It was left to Mirsky and Pollister⁶⁸ to make preparations from more favourable materials such as fish and bird erythrocytes and thymus, and to attempt to identify their product with actual chromosomes. The technique was briefly as follows: suspensions of red cells or of minced thymus were fragmented by brief treatment in the Waring blender, and cell debris and nuclei separated by a combination of straining and low-speed centrifugation; the nuclear fraction was then returned to the blender and the nuclei broken by a second blending; further straining

and centrifugation resulted in the separation of a fraction consisting of threadlike objects. These were identified as chromosomes on the basis of their positive staining reactions with such stains as aceto-carmin, orcein, and the Feulgen reagent, and on account of their resemblance to actual metaphase chromosomes. It must be borne in mind, however, that such threads were in all cases isolated from what must have been resting nuclei. Moreover, Lamb^{58a} has shown that the treatment described above causes thymus and erythrocyte nuclei to be deformed into thread-like formations which later break off isolated threads resembling chromosomes. Accordingly there is at present no definite basis for identifying such objects as chromosomes.

Chemical studies⁶⁹ of the above isolates have shown that these threads can be fractionated into a desoxypentose-nucleohistone, constituting about 90 per cent. of the mass of the thread, and a residual 'chromosome', which is a coiled thread consisting of a second protein with about 12-14 per cent. of pentose nucleic acid and about one-fifth as much desoxypentose nucleic acid. Further work remains to be done to determine the relationship between the physical and chemical organization of these isolated structures and that of chromosomes within the nucleus at different stages of the division cycle.

f. Other Cell Particulates

That the fractions described above do not exhaust the list of cellular constituents which may possibly be isolated by differential centrifugation is obvious. Indeed, others have been mentioned at the beginning of this section. Moreover, the supernatant fluid left after the separation of everything down to the dimensions of microsomes still contains abundant protein and enzyme activity²⁰, and includes particulate matter which centrifugation at still higher speeds would probably sediment. Mention may be made in this connexion of the macromolecular material isolated and studied by Stern and his collaborators from horse liver catalase preparations⁸⁴, and also the remarkable glycogen-protein complex isolated from liver by Lazarow⁵⁹, which opens up a promising field in the investigation of metabolic changes in cells by centrifugal methods.

It must be emphasized, in conclusion, that while the investigation of cellular extracts by centrifugal separation is a field in which rapid progress will undoubtedly be made, its central problem—that of the correlation of the properties of extracted fractions with definite aspects of cellular structure—has, to date, hardly been begun.

iv. THE HISTOLOGICAL LOCALIZATION OF ENZYME ACTIVITY

a. General

One of the most extensive and prolific fields in histochemistry is that of the demonstration of specific sites of enzyme activity within tissues.

Yet before 1939 only 3 groups of enzymes had been studied at all intensively—namely, the ‘peroxidases’* and ‘phenolases’*, and enzymes with some bearing on melanogenesis such as tyrosinase and dopa-oxidase. The methods employed for the demonstration of these enzymes have been critically considered by Lison⁶¹, and need not concern us further here.

b. Alkaline Phosphatase

In 1939 Takamatsu⁸⁷ and Gomori⁴³ almost simultaneously published methods for the histological localization of ‘alkaline phosphatase’ activity, and their description has been followed by a spate of papers, the bibliography of which now comprises over 60 references. ‘Alkaline phosphatase’ is an enzyme which catalyses the reaction



—that of hydrolysis of simple esters of orthophosphoric acid and phenolic or alcoholic hydroxyl groups^{25,62}. The pH optimum of this enzyme is in the region of 9.3, and it seems to correspond to enzyme A₂ in Folley and Kay’s classification of phosphomonoesterases³⁷.

c. Acid Phosphatase

Gomori’s alkaline phosphatase technique depends upon the principle that when tissue sections containing the enzyme are incubated in sodium glycerophosphate buffered at pH 9 in the presence of calcium ions, the phosphate ions released by the enzyme activity will be trapped and precipitated at the site of formation. The resulting white precipitate is visualized by treatment with cobaltous nitrate, when calcium is displaced and cobalt phosphate formed; this substance, on treatment with ammonium sulphide, is converted into black cobalt sulphide. Gomori⁴⁴ later adapted this technique to the localization of ‘acid’ phosphatase activity (pH optimum 4.5), and other authors have used similar methods to localize enzymes, splitting phosphate from a variety of phosphorylated compounds, including hexose diphosphate^{30,31}, adenylyl pyrophosphate⁴⁸, mononucleotides⁵⁷, and pentose and desoxypentose nucleic acids in both highly polymerized and relatively unpolymerized states⁵⁷. An essentially similar principle—i.e. the trapping and precipitation of one of the split products of an ester by a metallic ion, and the subsequent development of a coloured substance at the site, has been used to devise techniques for the demonstration of lipase⁴⁵ and cholinesterase activity⁴⁶.

* These do not correspond to the ‘oxidases’ and ‘peroxidases’ of the biochemist, but represent enzymes which are able to catalyse the oxidation of certain phenolic compounds either in the presence (peroxidases), or absence (phenolases), of hydrogen peroxide. All are demonstrated through the direct oxidation by the tissue of some aromatic base to a coloured compound.

d. Specificity

Granted that a given technique specifically demonstrates the activity of an enzyme, a number of further questions remain to be answered before the localization of precipitates, formed in sections following a whole sequence of chemical reactions, can be accepted as indicating the presence of enzyme activity at these sites. These questions have been ably formulated by Danielli^{25,26}, and are as follows: How much enzyme is destroyed by the experimental procedure, and, if there is destruction, does this take place to the same degree at all sites, or selectively at certain sites?; are the compounds formed all deposited at the original site of enzyme activity or at sites which have a special affinity for them?; is the enzyme in a fixed preparation in its physiologically normal position? These questions must be answered for every projected enzyme technique before its results can be fully acceptable.

In the case of alkaline phosphatase, a careful and critical study of the technique²⁵ has given a satisfactory answer to these questions. A large amount of the enzyme is destroyed by the experimental procedure, but apparently not selectively: sites with specific affinity for calcium phosphate exist in sections, but these seem to coincide with sites of phosphatase activity; the fact that enzyme activity is found at the same sites following widely different methods of fixation^{25,34} renders it probable that these locations are the enzyme's normal position. Moreover it is possible that some substance involved in the reaction can diffuse within sections during incubation.^{64a} The results of the technique are thus hard to interpret unless adequate controls are routinely used. Lison⁶² recommends three such controls: one without substrate; and two in which the enzyme is inactivated by heat or M/100 KCN. A positive difference between the experimental and control sections indicates alkaline phosphatase activity. Conversely, the absence of such a difference does not indicate a total lack of alkaline phosphatase. The amount present may initially have been small enough to suffer complete inactivation by the processes of fixation and embedding.

By the above standards the technique for acid phosphatase is far less satisfactory. Several authors^{2,50,62,64} have found the technique to be capricious and unreliable, sections from the same block mounted side by side often showing inexplicable differences in staining. This is in part due to the low affinity of the enzyme for the sodium glycerophosphate commonly used as substrate, and also in part to the lability and perhaps solubility of the enzyme⁷⁴. Acid phosphatase is inactivated to a greater extent by the commonly used acetone or alcohol fixation than is alkaline phosphatase; the enzyme activity remaining in paraffin embedded tissues is approximately 5 per cent. in the case of acid, but 20–30 per cent. in the case of alkaline phosphatase⁸³. There is also evidence that acid phosphatase can be leached out of, or moved about in, acetone

fixed tissues by water, as can the precipitate of lead phosphate used to visualize its activity². Moreover, little correlation can be found between acid phosphatase activity as measured chemically, and that revealed histologically⁵⁰. A full critical analysis of this technique will be needed before full confidence can be placed in its results.

None of the other enzyme localization techniques proposed^{45,46,48,72} has been critically investigated, although one of them⁴⁸ has been severely criticized on chemical and physiological grounds⁷⁰. Further work will therefore be necessary before the value of these techniques is established. The study of 'substrate-specific', rather than 'bond-specific' phosphatases⁶² is, however, only in its infancy, but its results are extremely promising. The varying reactivity towards different substrates shown by sites in the same tissue points to the existence of a number of 'specific' enzymes^{30,31}. Identification of the enzymes shown by histochemical techniques with those described as a result of purely biochemical techniques, however, has not yet been made, and much new work is needed on this question.

V. THE USE OF ENZYMES AS HISTOCHEMICAL REAGENTS

a. *General*

Enzyme preparations have been used in cytology in two main ways: first, as macerating agents and to improve the clarity of cytological preparations³⁵; and second, as specific reagents in cytochemical tests. Only this second aspect of their use will be discussed here.

The classical example of the use of an enzyme as a cytochemical reagent is the use of saliva to distinguish between glycogen and 'amyloid'³. Both substances are coloured similarly by iodine, but only glycogen is removed by the action of salivary amylase. Examples of the use of other enzymes are fairly plentiful in the earlier cytological literature^{39,65,89}, and some of them have been criticized by Lison⁶¹.

b. *Ribonuclease*

In general, however, the majority of these techniques are unreliable on account of the considerable lack of specificity of the enzyme preparations, and up till 1940 little use was made of enzymic destruction as a cytochemical tool. Recent years, however, have seen a great revival of interest in the cytochemical use of enzymes, principally in connexion with the cytological localization of the nucleic acids. This followed the isolation and crystallization by Kunitz⁵⁸ of ribonuclease, a highly specific depolymerase for the pentose nucleic acids, and the almost simultaneous publication by Brachet^{6,7} of a cytochemical test for pentose nucleic acids based upon the treatment of sections with a pancreatic extract containing this enzyme. Modifications of the Brachet test, using the Kunitz enzyme, have been extensively employed by

cytologists^{29,41,71,77}. Similar identification of desoxypentose nucleic acid¹¹ has been attempted by means of the highly purified pancreatic desoxyribonuclease first described by McCarty⁶³, and also by means of spleen thymonucleodepolymerase. Moreover, a test for the differential identification of the two types of nucleic acid by means of these enzymes has been described⁷⁸. The application of similar methods for the identification of polysaccharides has been proposed⁴⁹. At the present time, therefore, it seems appropriate to consider the bases for the validity of such techniques.

c. Specificity

A common principle underlying all cytochemical tests in which enzyme preparations are used is that by treating sections with an enzyme those parts of a cell are removed which constitute the specific substrate for the enzyme in question. Thus, for such a test to be completely acceptable it must satisfy the following criteria²⁶: (1) an enzyme preparation must be used whose activity is limited to a single substrate; (2) the specific substrate must be so arranged that it is accessible to attack by the enzyme; (3) enzyme treatment must remove only this substrate from the treated cell.

As Danielli^{26,27} has pointed out, none of the techniques so far used satisfy these criteria absolutely. Both crystalline ribonuclease and pancreatic desoxyribonuclease possess some proteolytic activity, for example⁶³. Indeed, as Pirie⁷⁶ has pointed out, it is hard to find a single enzyme preparation, however highly purified, whose activity is restricted to a single substrate. As regards the second criterion, we do not know to what extent protective shells of protein, or alterations in the physical state of such high polymers as the nucleic acids, can protect them from the action of enzymes. For example, it is well known that treatment with desoxyribonuclease fails to modify the basiphilia of sperm heads⁹, and therefore, presumably, does not remove the desoxypentose nucleic acid which they are known to contain in high concentration. A third possibility has also to be considered: where a part of a cell is removed by an enzyme we cannot state definitely that it consists of the substrate for the enzyme used. It may consist almost entirely of protein, bound to the rest of the cell by a small quantity of the enzyme substrate under consideration.

Though the above criticisms are serious ones, they do not constitute grounds for the total abandonment of cytochemical methods involving the use of enzymes. Indeed, much of their force is lessened when we consider the results of enzymatic methods in their proper setting—i.e. in relation to information obtained by other accepted cytochemical methods, and by the grosser, but more quantitative, techniques of classical biochemistry.

While there is no doubt that even highly purified enzymes are in a sense non-specific as to substrate, precautions can be taken to restrict their action as far as possible to the substrate under investigation. Ribonuclease has a peculiar advantage in this respect as it is relatively heat-stable. The small amount of proteolytic activity which characterizes most preparations can be removed by heating for a few minutes at 80°C. In the case of desoxyribonuclease the proteolytic activity can be largely eliminated by dilution.

An integral part of the enzymatic technique for the identification of nucleic acids is the staining of enzyme-treated and control sections with basic dyes such as pyronin⁷. While staining with basic dyes by no means constitutes a cytochemical technique in itself, yet the number of different classes of compounds in tissues which show basiphilia is small. Thus while removal of an unspecified part of a cell by ribonuclease is no evidence that that part consists largely of nucleic acid, the removal of the *basiphilic property* of a cellular region by this enzyme is evidence that this part of the cell owes its basiphilia to the presence of pentose nucleic acid. In fact the kind of stains used and the reactions developed to them are as important a part of the technique as the enzyme treatment.

In addition, comparison of the ultraviolet absorption spectra of regions of cells before and after ribonuclease treatment shows that ribonuclease selectivity abolishes at certain cytoplasmic sites the characteristic strong U.V. absorption at 2,600 Å. due to purine and pyrimidine containing compounds^{41,56}. These same cytoplasmic areas are also invariably basophilic, and the presence of pentose nucleic acids can generally be shown in these tissues by gross biochemical methods. To deny, on the basis of the above criteria, that sites with the combined properties of purine-pyrimidine absorption and basiphilia, both of which are removed by ribonuclease, represent sites of localization of pentose nucleic acids, is to show a timidity not normally characteristic of cytologists.

It therefore appears that the enzymatic techniques for the localization of nucleic acids in sections, while themselves not rigorous, are nevertheless valuable adjuncts to other cytochemical methods, but should only be relied on to give data of a confirmatory nature. Even so, they are so beset with pitfalls that it is suggested that certain principles should be adhered to in their use if unreliable results are to be avoided. These are:

1. Only crystalline or highly purified enzyme preparations should be used, with precautions designed as far as possible to restrict their action to a single substrate.
2. The cell destruction produced by the enzyme should be considered only in relation to some cellular property (basiphilia,

characteristic U.V. absorption) which has at least some specificity on its own account.

3. The reaction should only be performed on tissues fixed in such a way as to preserve the substrates in a form which is still susceptible to the action of the enzyme. All steps in the technique should be carefully controlled and standardized, and a control slide incubated in distilled water included to check on possible solvent action of the buffers used⁸⁶. As shown by Catcheside and Holmes¹¹, enzyme reactions performed on fresh tissues are often capricious and difficult to interpret.
4. Negative results of the technique should be neglected. For example, removal of the basiphilic property of a nucleus by desoxyribonuclease confirms the presence of desoxypentose nucleic acid in the nucleus in question; the failure of the reaction does not show that this substance is absent.
5. The results of enzyme tests on sections should only be considered in relation to evidence obtained by other methods.

vi. MICRO-DISSECTION

In spite of the great usefulness of micro-dissection techniques in many fields of biology, they have not yet been universally applied. In most instruments the movements of the microneedle are brought about by screw and lever mechanisms resembling the fine adjustment of a microscope. The Chambers¹² and Peterfi⁷⁵ designs employ three such mechanisms with directions of movement mutually perpendicular to one another, and adjustment of three separate controls is thus necessary to bring the needle to a predetermined position in space. Considerable skill born of long practice must be acquired to do this with ease and precision, and the labour involved may have mitigated against the universal adoption of the method. Recently, however, an entirely new instrument working on pneumatic principles has been developed³⁸, which does away with many of the disadvantages of the older instruments, and which can be used successfully by relatively unskilled operators. The microneedle is moved by the deformation of one or more of three tambours by air pressure. The tambours are connected by rubber tubes to three pistons and cylinders set at right angles to one another. The movement of these pistons is controlled by a single lever resembling the joystick of an aircraft. Movement of this lever in a horizontal plane moves one or both of the horizontal pistons, while the vertical one is moved by rotating the handle. With a little practice something like an independent movement of the microneedle in all three planes can be achieved with this single control lever. Such an instrument, by reason of its remote control, is free from vibration, and thus need not be massively constructed. The absence of screws and levers also makes it free

from backlash. A variable range and sensitivity of movement of the tip of the microneedle, in conformity with the size of the field of view at different powers of the microscope, has also been achieved. A sliding collar is fitted around the vertical control handle and connected by rods to the pistons which move the needle in a horizontal plane. A movement of this collar alters the range of movement of the piston levers, and thus the range and sensitivity of movement of the microneedle.

The chief disadvantages of this instrument at present are its high cost and delicacy of construction. Doubtless the future will see the development of cheaper and more robust instruments embodying similar principles. Since this article went to press such an instrument has indeed appeared^{1a}.

A second instrument introduced by de Fonbrune³⁸, the microforge, greatly simplifies the manufacture of the glass instruments necessary for micro-dissection work. Whereas previously the drawing of adequate pipettes and microneedles required great skill, while it was not possible to produce instruments of a given calibre at will, the use of the microforge enables pipettes and needles of standard calibre easily to be made. Manufacture of more complex instruments, such as microscalpels, is also possible.

The micro-dissection work of Chambers and his school has given us considerable information as to the physical state of cells and tissues, and the behaviour of cell organelles when released from the cell into an extraneous medium. With the new instruments available, considerable progress will probably be made with such techniques as micro-injection, in order to find out as much as possible of the chemical organization of the interior of cells. In order that this may be done, it will first of all be necessary to develop a neutral medium which can be injected into cells without altering their organization. A promising beginning in this direction has already been made¹³.

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CHAPTER I (*cont.*)

CYTOLOGICAL TECHNIQUES

C. MICROSCOPY

By R. BARER

i. INTRODUCTION

FEW methods of cytological research are as satisfactory as that of direct observation. Much can be inferred by indirect physiological and chemical methods, but in the long run such inferences must be correlated with the structure of the cell as observed by means of the microscope. It would be impossible to cover all aspects and methods of microscopy in a short article. Apart from a brief discussion of a few selected fundamental principles we shall be concerned here mainly with the potentialities and limitations of certain new methods of microscopy. For a more detailed account of routine microscopy the reader is referred to such works as those of Barnard and Welch¹⁵, Beck²¹, and Shillaber¹⁰⁵. The last-mentioned is a modern work containing a wealth of valuable information, which should be accessible to all serious microscopists.

ii. SOME GENERAL PRINCIPLES OF MICROSCOPY

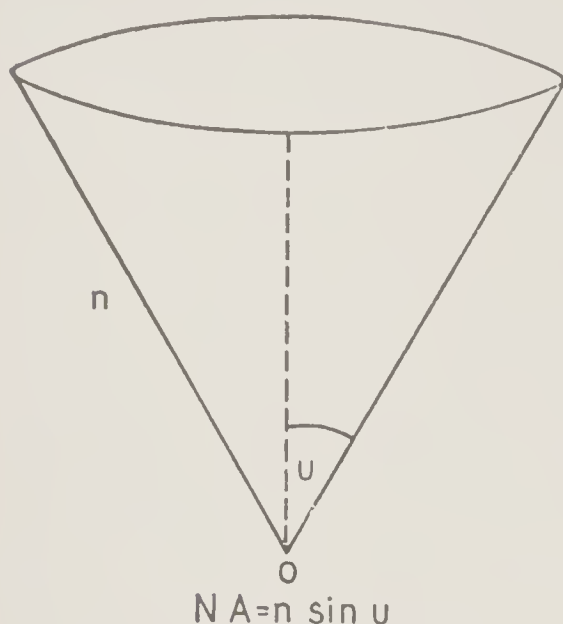
In general there are two main aims in all methods of microscopy. (1) The formation of a magnified image as free as possible from optical defects or aberrations.

At first sight this might seem to be all that is required, but a moment's reflection shows that this is by no means the case. Many objects, for example living cells, which are of particular interest to the cytologist, would be almost invisible if examined under a microscope designed to fulfil this first condition alone. Mere magnification is not necessarily enough; what is required is (2) Contrast, i.e. a difference in intensity or colour between various points in the object and the background. It is this factor which determines whether the magnified image can be seen. The human eye and the photographic plate are sensitive to intensity or amplitude variations in the incident light, and all methods of microscopy depend on different means of producing such variations.

a. Numerical Aperture and Resolution

The resolving power of a microscope, or the ability to distinguish two close points as separate, is not a question of magnification alone. There is a limit of magnification beyond which no new detail can be observed. The ability to resolve detail is governed by an optical constant of the

system known as the Numerical Aperture (NA). This concept was introduced by Abbe in 1873. In Text-fig. 1 a cone of rays of light enters



TEXT-FIG. 1. Numerical aperture.

a lens from a point O . Let u be the semi-angle of this cone, and n the refractive index of the medium. Then the NA is defined as $n \sin u$. The NA of the objective lens and condenser is a matter of fundamental importance in microscopy. In general the NA of the condenser should be approximately equal to that of the objective, though in practice it is often slightly less. The *working* NA of the system is the mean of the NA of the objective and that of the condenser. Several properties of the optical system depend on the NA.

1. The resolution is given by $R = k\lambda/NA$ where λ is the wavelength and k is variously given as 0.5 (Abbe) or 0.61 (Rayleigh). Thus the greater the NA the greater the resolving power.
2. The amount of light transmitted by a lens is proportional to the square of the NA.
3. The depth of field decreases as the NA increases.

It will be noticed that the expression $n \sin u$ for the NA of a lens includes the term n , the refractive index of the medium between the lens and the object. If more than one medium is present, the NA is determined by the least refractile medium. Thus if there is a layer of air between object and objective the NA *cannot possibly exceed 1.0*, no matter what the nominal rating of the lens may be. Similarly, if the object is immersed in water the NA cannot exceed 1.33. The highest NAs are obtained by the use of immersion oils, chosen to match the lens material in refractive index and dispersive power. The highest NA in general use in a microscope objective is 1.4, though lenses have been designed (for immersion in monobromide of naphthaline) with an NA of 1.6. Similar considerations apply in the case of the condenser. No condenser can have an NA greater than 1.0 unless there is a layer of oil or other liquid between it and the object. If an objective of NA 1.4 is used in conjunction with a dry condenser the maximum working NA is reduced to

$$\frac{1.4 + 1.0}{2} = 1.2.$$

For detailed cytological work involving the highest resolution an oil immersion condenser is essential.

We have seen that there is a limit of magnification beyond which no

new detail can be resolved. This limit is theoretically 250 times the N.A. However, this formula presupposes perfect eyesight and in practice a limit of 1,000 times the NA is usually taken. Greater magnifications than this usually result in some loss of definition in the image.

b. Lens Aberrations

No lens produces a perfect image. Apart from theoretical considerations the performance of a lens is limited in practice by a number of factors called aberrations. These can be divided into those due to the lens *material* and those due to the lens *shape*.

c. Chromatic Aberration

Is due to the fact that the refractive index of the lens material varies with the wave-length of the light employed. One result is that blue rays are brought to a focus closer to the lens than are red rays. This effect is known as longitudinal chromatic aberration, or chromatic error of focus. Another manifestation of chromatic aberration is lateral chromatic aberration, or chromatic error of magnification which results in a lens having different magnifications for different wave-lengths.

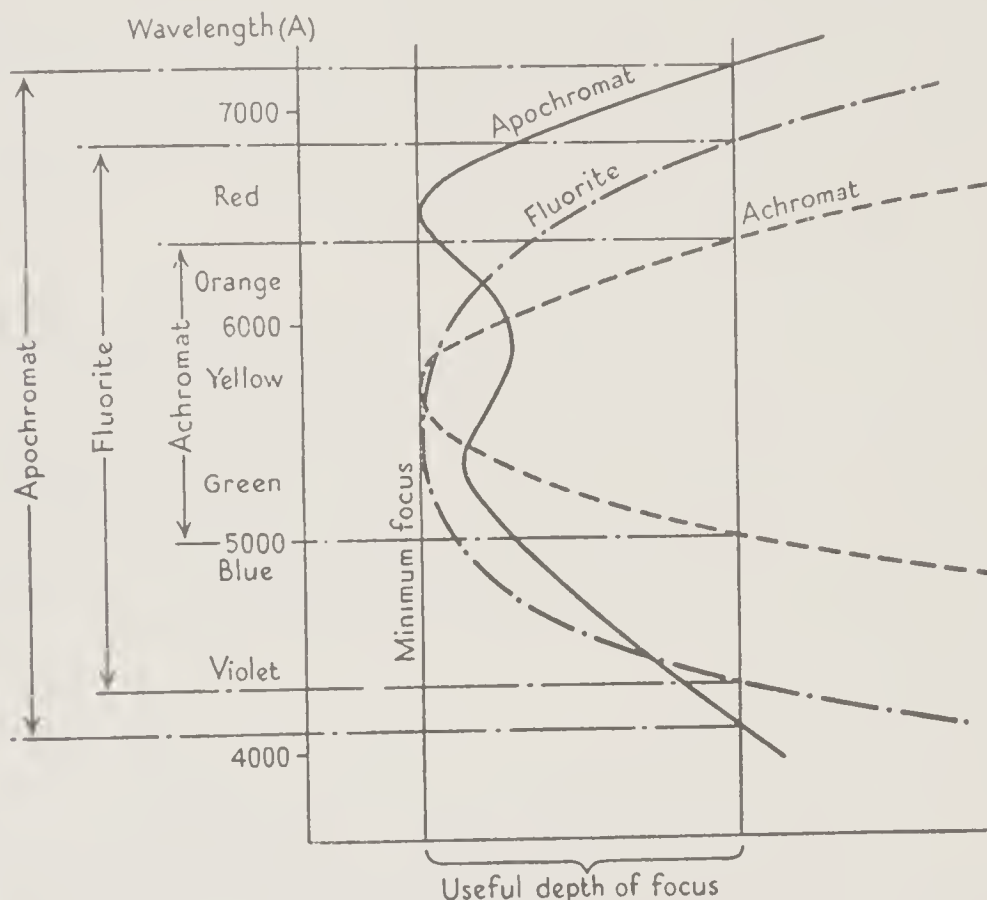
d. Spherical Aberration

Is the most important of those aberrations due to lens form, the others being coma, astigmatism, curvature of the field, and distortion. It is due to the fact that rays passing through peripheral zones of a lens do not in general come to the same focus as rays passing through the central zones. This results in loss of light and blurring of the image.

e. Correction of Aberrations

It is possible to minimize or even eliminate spherical aberration at certain wave-lengths by suitable design of lens shape (e.g. aspherical lenses) or by the addition of correcting lenses of proper curvature. The latter is the method usually adopted in the case of microscope objectives. The problem of chromatic aberration is impossible to solve completely with lenses, and all that can be achieved is a useful compromise over a more or less narrow band of wave-lengths. One method is to use a monochromatic objective, i.e. one corrected for spherical aberration at a certain wave-length, in conjunction with monochromatic light of that wave-length. Such objectives are used with the quartz ultraviolet microscope. They are usually meant to be used at 2,750 Å.U. If used at other wave-lengths it is necessary to refocus, and the introduction of spherical aberration results in some deterioration of the image, particularly at the periphery of the field. In the visual range so called 'achromatic' objectives are frequently used. These combine glasses of different refractive indices in such a way as to bring light of two different colours to a common focus. They are usually corrected for the C and F lines

(i.e. 6,563 Å.U. and 4,861 Å.U.), which means that wave-lengths within this range contribute to the image. Spherical aberration is corrected for an intermediate wave-length, usually about 5,600 Å.U., in the yellow-green. Thus for the best results such lenses should be used with a yellow-green or green filter. Such filters are very useful. The colour is



TEXT-FIG. 2. Colour corrections of typical achromatic, apochromatic, and fluorite objectives. The range of wave-lengths usefully contributing to the formation of the image is shown.

(Courtesy of Messrs. Cooke, Troughton, and Simms Ltd.)

restful and pleasing to the eye, which is very sensitive in the yellow-green region. Glare is reduced, and contrast is improved with many routine biological stains.

The best-corrected objectives now obtainable are known as *apochromats*. These were introduced by Abbe in 1886 and contain fluorite and other special glasses. Their performance is greatly superior to that of the achromats since they are chromatically corrected for three colours, usually the C, F, and G (4,308 Å.U.) lines, and are spherically corrected for two colours. In practice apochromats should yield excellent images throughout most of the visible spectrum, even without the use of colour filters. They should be used with compensating eyepieces in order to eliminate residual chromatic error of magnification. Apart from price, the chief disadvantages of the apochromats are the greater curvature of the field of view which they produce and the fact that they require very critical adjustment for optimum performance. For visual work involving the very utmost in resolution they are supreme, but for many

purposes a correctly used achromat, together with a green filter, will give an image which is almost as good. Another range of objectives, often called semi-apochromats, is manufactured by some firms (e.g. the Cooke fluorite objective and the Watson Holographic series). These have corrections intermediate between those of the achromats and apochromats, and give an excellent performance when used with a green filter. Text-fig. 2 shows colour curves for typical achromatic, fluorite, and apochromatic objectives.

f. The Condenser

It is probably true to say that no component of the microscope is so frequently misused as the substage condenser. Many medical and biological students and even research workers appear to regard the condenser and its iris diaphragm solely as a means of controlling the intensity of illumination. Nothing could be farther from the truth. The corrections of a condenser need not be as good as those of an objective, but they should not fall far short. Moreover, the condenser must be used properly. There is in general only one correct setting for the condenser and its diaphragm for a given illuminating system and objective. Only very minor deviations from this setting are permissible. The condenser should not be 'racked down' in order to obtain more even illumination or to reduce glare or intensity. Nor should the substage iris diaphragm be used for similar purposes. Its sole function is to limit the effective NA of the condenser. It is true that reducing the diameter of this diaphragm often improves contrast, but only at the expense of resolution. The diaphragm should be adjusted to such a size as to supply a cone of light of NA equal to (or in practice usually slightly less than) that of the objective.

There are three types of condenser in general use. The uncorrected Abbe condenser is mentioned only to be condemned. It is quite useless for high-resolution work in cytology. Its few virtues include cheapness and ease of adjustment. The aspherical aplanatic condenser is capable of giving excellent results, but is not chromatically corrected and is best used with a green filter. The achromatic-aplanatic condenser is undoubtedly the best for critical work and should be used wherever possible. To feed an expensive apochromat by means of an Abbe condenser would be like running a high-speed aeroplane on crude fuel. Apart from defective illumination and loss in resolving power the use of an uncorrected or wrongly adjusted condenser manifests itself chiefly by glare and loss of contrast. A basic principle in all methods of illumination for microscopy is that the area of object illuminated should not be greater than the field of the objective. If this condition is not adhered to, stray light may enter the objective from parts of the specimen not in the field of view, with resultant glare. A poorly corrected condenser

usually illuminates areas outside the field of view, and produces excessive stray light.

g. Methods of Illumination

The theory underlying the formation of images by the microscope is far from simple (see Rayleigh⁹³, Martin⁷⁵). At one time it was believed that an approach to a perfect image could only be made if the object were self-luminous. This is only rarely the case and in practice most specimens are illuminated by 'borrowed' light. It was suggested that the object could be made to behave as a self-luminous body if an image of the source of light were focused on it. This was the basis of 'critical' illumination. It is now known that this condition is unnecessary and, indeed, is unattainable in practice (Hartridge⁶⁰). The essential feature of any method of illumination is that as large an area as possible of the objective aperture should be uniformly filled with light. Ideally the entire aperture should be filled, but in practice glare limits the illumination to between two-thirds and nine-tenths of the aperture. The Köhler method of illumination (Köhler⁶⁷) and its modifications are particularly recommended. An image of the source is focused by means of a suitable lens on to the diaphragm of the substage condenser. The image of a field stop (usually, but not necessarily, an iris diaphragm situated at the lamp) is then focused by the condenser on to the plane of the specimen. If the image of the source is large enough to fill the condenser aperture, the objective aperture should be uniformly filled with light. The condenser aperture diaphragm should be adjusted until between two-thirds and nine-tenths of the objective aperture is illuminated. The importance of the field stop cannot be over-emphasized. It offers a convenient method of controlling the size of the source and is a most potent factor in reducing glare. The image of the field stop should not be greater than is required to fill the field of view and may frequently be made much smaller in order to improve contrast. The proper use of the field stop may make all the difference between visibility and invisibility of difficult details.

iii. METHODS OF MICROSCOPY

a. Conventional Microscopy

This refers to the ordinary use of the lens microscope for studying stained and unstained specimens. We have already seen that this method is liable to prove very unsatisfactory with living unstained cells owing to lack of contrast. Fixation followed by staining is resorted to as a method of improving contrast. This is an unfortunate necessity as artefacts are inevitably produced. Gutman and Young⁵¹ have expressed the matter very well in discussing the structure of the muscle end-plate.

In considering the relationship it must always be kept in mind that the tissues have been fixed and stained, which cannot fail to have altered the appearance and, indeed, are designed to do so. The object of fixation and staining is not to discover the *appearance* of the living organs which we know to be transparent. What the biologist does wish to know is the relationship between the nerve as a conductor, the end-plate as a conductor and, if possible, the muscle as a contractor. The fixation and staining of the preparation and the looking at it are experiments designed to clarify these relationships.

We shall not discuss this method of microscopy further since it is very adequately dealt with in many standard works^{15,21,105}.

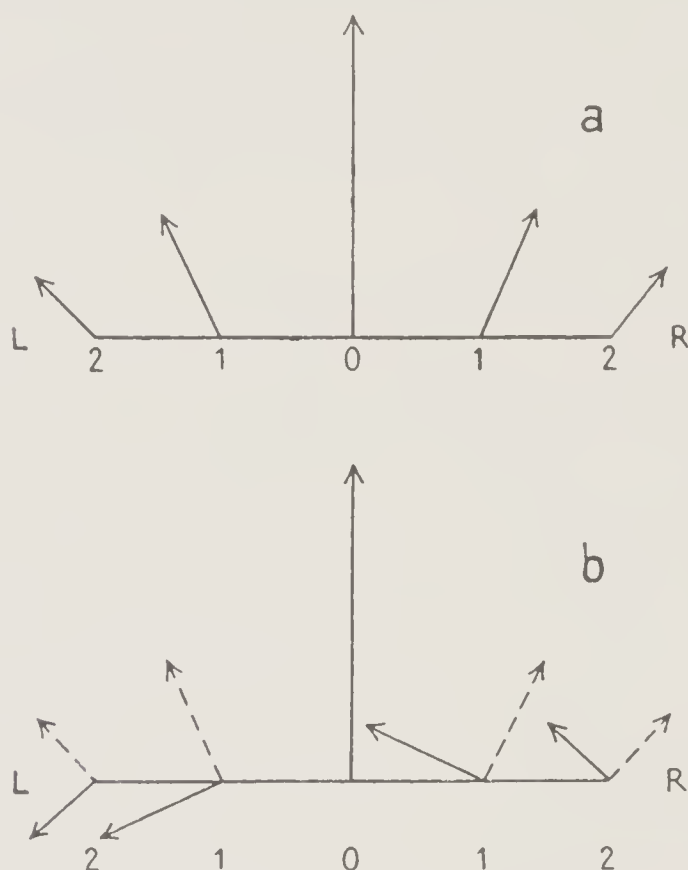
b. Phase-Contrast Microscopy

We have already seen that the human eye and photographic plate are sensitive to amplitude or intensity changes in light. An object such as a living cell is highly transparent and produces very little intensity change in the transmitted light. This does not mean that the latter is unaltered. On the contrary, every variation in thickness and refractive index in the object produces a *phase* change in the incident light. A simple example will serve to illustrate the fact that the eye cannot appreciate phase changes. When we look through a transparent window or a pair of spectacles, the incident light is retarded in passing through the glass so that it arrives at the eye a fraction of a second later than it otherwise would. This light is said to have undergone a phase alteration due to the refractivity of the glass. Yet a clean, homogeneously transparent window is quite invisible in the absence of reflections. Clearly, what is required for the study of living cells and other transparent material is a method of microscopy which converts phase variations into intensity variations. This is precisely what is achieved by phase-contrast microscopy. The method was first conceived by Zernike¹¹⁶ and used for the purpose of testing telescope mirrors. Zernike¹¹⁷ later adapted the method to microscopy. Important pioneer work was carried out in Britain by Burch (Burch and Stock³¹) before commercial equipment became available. The Zernike system was developed by the firm of Zeiss (see Köhler and Loos⁷⁰) with good results.

At the time of writing phase-contrast equipment is put on the market by Cooke, Troughton, and Simms in this country and by the American Optical Company (Spencer) and Bausch and Lomb in America. Intensive research is going on in a number of laboratories, and several firms, including Watsons and Beck, intend to produce their own models.

As regards the theory of the method, it must be stressed that it is impossible to give an adequate explanation without the use of mathematics or at least without accepting the results of the mathematical argument. Those interested should consult two important papers by

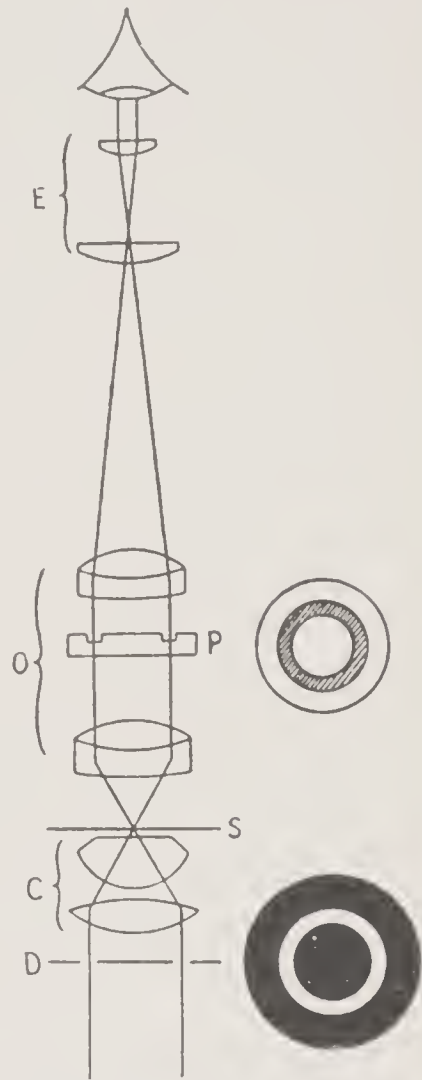
Zernike¹¹⁷. The writer has given a brief non-mathematical summary of the essential argument of this work in a recent article (Barer⁸). The papers of Burch and Stock³¹, Martin⁷⁶, and Bennett *et al.*²² should also be consulted. Briefly, the theory of the microscope shows that the final image can be regarded as the result of interference between the direct (geometrical) image and a number of lateral or diffracted images. For



TEXT-FIG. 3. Vector representation of (a) amplitude grating; (b) phase grating.

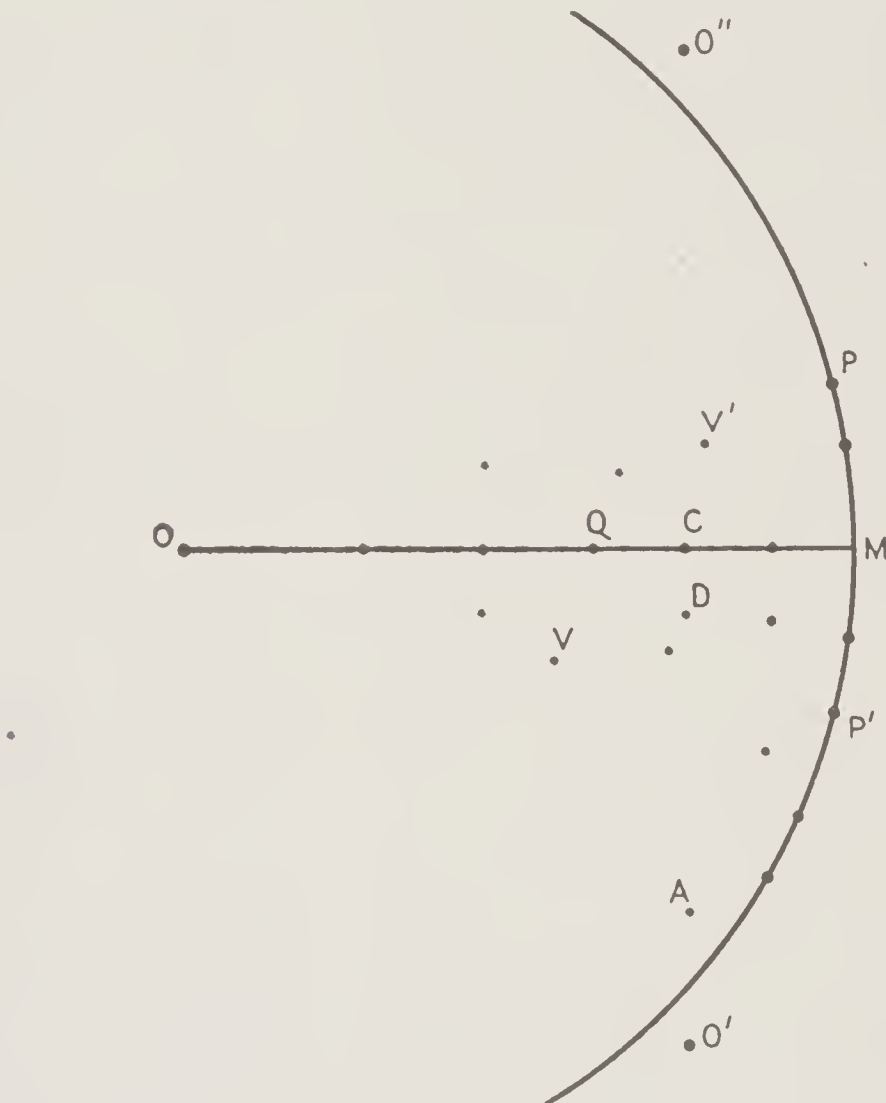
simplicity, we may take as the object a grating composed of a large number of close parallel bars. Two sorts of grating can be considered. One in which the *absorption* of alternate bars differs—an amplitude grating, and the other in which alternate bars are equally transparent but differ in refractive index—a phase grating. It can be shown that the final image can be represented diagrammatically as being composed of a number of vibrations or vectors. In Text-fig 3 (a) the vectors representing the image of the amplitude grating are shown. Text-fig. 3 (b) shows the representation of the image of the phase grating. The first grating will be visible, the second will generally be invisible or only faintly visible. Now according to the mathematical analysis the two sets of vectors in Text-figs. 3 (a) and 3 (b) differ only in that the lateral vectors are rotated 90° relative to each other. The central vectors are identical. If, therefore, it were possible to rotate the lateral vectors of Text-fig. 3 (b) by 90° without affecting the central vector, the result would be that the phase grating would appear to the eye as if it were an amplitude grating! This remarkable result is achieved in practice

by an arrangement which separates the geometrical image from the diffracted images. In order to do this it is necessary to have a diaphragm of convenient shape such as a linear slit or an annulus, situated at the front focal plane of the substage condenser. An image of this diaphragm is formed at the back focal plane of the objective. If an object is present between the condenser and objective, a number of diffracted images of the diaphragm will also be formed as well as the direct image. In general, however, the diffracted images will overlap considerably and may not be individually distinguishable (see Conrady⁴⁰, Barer⁸). A *phase plate* is now placed at or near to the back focal plane of the objective. This consists essentially of a transparent disk containing a groove (or an elevation) of such a shape and size as to coincide with the direct image of the substage diaphragm (Text-fig. 4). The depth of the groove or elevation and the refractive index of the plate are so arranged as to cause the rays forming the direct image to differ in optical path by one-quarter of a wave-length from those forming the diffracted images. A quarter of a wave-length change in path is equivalent to a 90° change of phase, so that by separating the direct and diffracted images and making them pass through different regions of the phase plate, we have achieved the theoretically desired condition. The extension of the mathematical theory to objects other than gratings is difficult, but it is possible to represent a more general type of object by another type of vector diagram. In Text-fig. 5 the vector OM represents a vibration which has passed through the object without suffering either a change of amplitude or phase. Vibrations which have only suffered a change of amplitude can be represented by vectors such as OQ along OM , the length OQ being proportional to the amplitude. Vibrations which have been altered in phase, but not in intensity, will be represented by vectors such as OP, OP' , whose terminal points lie on the circumference of the circle with OM as radius. The amount of phase change is measured by the angle POM . Vibrations which have undergone changes in both phase and amplitude will be represented by vectors such as OV , where the length OV represents the amplitude and the



TEXT-FIG. 4. Diagram showing method of producing phase-contrast illumination. *D* annular substage diaphragm. *C* condenser. *S* object stage. *O* objective lens. *P* phase plate with annular groove. *E* eyepiece.

angle VOM the phase change. The direct undiffracted image can be represented by the vector OC which is the mean of all the vectors representing the object. The effect of the phase plate is to turn this vector through 90° , which is equivalent to shifting the origin from O to O' (positive phase contrast) or to O'' (negative phase contrast). Although it may not be obvious at first sight, this method of representation is almost identical with that given by Martin⁷⁶. It is a very useful and



TEXT-FIG. 5. Vector representation of a general type of microscopical image (see text).

simple method, and enables many practical points to be understood. Let us first consider an object composed almost entirely of phase changing, non-absorbing material, such as occurs in a highly transparent living cell. Let the phase changes be small. Such an object will be represented by points such as P , P' on or very near to the circumference, close to M (Text-fig. 5). With ordinary illumination, O is the origin and the intensities of the vibrations are proportioned to $(OP)^2$ and $(OP')^2$, &c. (since the intensity is proportional to the square of the amplitude). These quantities are all very nearly equal so that we should expect very little contrast. If, on the other hand, we use phase-contrast illumination and have O' as our new origin, the intensities are proportional to

$(O'P)^2$, $(O'P')^2$, &c. These quantities may differ by quite considerable amounts, so that good contrast will result. With O' as origin, areas of high refractive index will appear darker than those of low refractive index. With negative phase contrast (origin at O'') the reverse is true. For small changes of phase, the relationship between intensity and refractivity will be roughly linear. This vector diagram brings out another useful point. If it were possible to bring O' to some point such as A nearer to the group of points representing the object, then the ratio of $(AP)^2$ to $(AP')^2$, i.e. the contrast, would be increased. This can be achieved in practice by using a partly absorbing phase plate, and is a valuable method of increasing contrast in difficult cases. It will also be appreciated that while the 90° phase plate may give the best results in many cases, there is no real reason why this value should be rigidly adhered to. With some types of object other phase changes may be more suitable. Indeed, ideally we should like to be able continuously to vary both the phase and amplitude (absorption) characteristics of the phase plate. Care must be taken, however, to ensure that the new origin is not shifted to some point such as D , within the main cluster of points. If this does occur some points will appear with reversed contrast, i.e. a dark-ground effect will be produced. It is frequently stated that phase-contrast illumination is of no value for the study of stained or absorbing material. This is a sweeping generalization based on a rather special case. If the object were composed *entirely* of absorbing points (represented by vectors on OM) then it would be true that shifting the origin from O to O' would result in loss of contrast. However, most stained biological material consists of points (V , V') which both absorb and refract light. In such a case, contrast may be improved, particularly in areas which have remained unstained or very lightly stained (Barer⁶). It is always worth examining a stained section by phase contrast illumination, as extra details are frequently revealed. It must be remembered, however, that staining usually exhibits some degree of specificity, whereas phase-contrast is non-specific and renders visible all refractile changes of sufficient magnitude. This non-specificity may be very confusing with certain types of material, e.g. nervous tissue (Barer⁶). Some living cells are so full of cytoplasmic granules as to obscure the nucleus, and it is rarely possible to predict the suitability of any given material for examination by phase-contrast microscopy.

Apparatus. The standard equipment as marketed by Cooke, Troughton & Simms is similar to the original Zeiss model, and consists of a set of objectives each containing a fixed phase plate, and a condenser with interchangeable substage annuli (Taylor¹¹⁰). This apparatus has the merit of being simple, is easily adapted to almost any standard microscope, and is very efficient for most work. As we have seen, however, it is sometimes useful to be able to vary the phase and amplitude

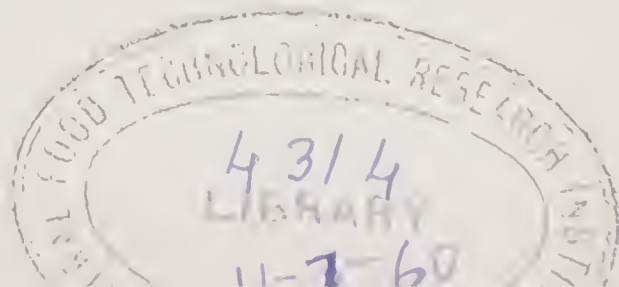
characteristics of the phase plate. The American Optical Company produces a special set of objectives with a variety of interchangeable phase plates (Bennett *et al.*²²; Richards⁹⁶). This makes the apparatus rather more elaborate and expensive, and seems scarcely warranted for ordinary routine use, though most valuable to the research cytologist. More elaborate methods of obtaining *continuous* variation of phase and amplitude have also been described (Osterberg⁸⁴; Hartley⁵⁹; Taylor¹¹¹; Kastler and Montarnal⁶⁶). These depend on the use of polarized light and an arrangement whereby the direct and diffracted images are polarized differently so that the desired phase difference between the two can be controlled by means of a birefringent plate or compensator (see section on the polarizing microscope). Such apparatus is rather cumbersome and presumably could only be used for the study of a non-birefringent object, so that it is greatly to be hoped that other methods of achieving the same result will be developed.

Although the commercial equipment is convenient and efficient it is possible for any keen amateur to construct his own phase contrast apparatus. Instructions for making phase plates have been given by Burch and Stock³¹, Zernike¹¹⁷, and Salmon⁹⁹. The commercial equipment is preferable since it enables the user to change over rapidly from one objective to another and from normal to phase-contrast illumination.

The method of using the microscope for phase-contrast illumination differs in no essential respects from that for other methods of microscopy. Some form of Köhler illumination is to be preferred both for visual and photographic work. The correct use of the field stop is particularly important for improving contrast. The intensity of illumination is generally rather less than with normal illumination and a fairly intense source is necessary for high-power work. A low voltage, high wattage tungsten lamp is quite suitable. There is a common belief that since the function of the phase plate is to introduce a quarter of a wavelength change of phase, only monochromatic light may be used. This is erroneous. As we have seen, it is not essential for the phase change to be exactly one-quarter of a wave-length, and indeed other values may be more desirable on occasion. In practice, approximately monochromatic green light yields the best results and the advantages of a green filter for use with achromatic objectives apply as much to phase-contrast illumination as to other methods, but there is no great objection to the use of 'white' light or other colour filters. It is often stated that the method can only be used for very thin objects such as single cells. While it is true that such material gives the best results, it should be realized that it is *transparency* rather than *thickness* which is the limiting factor. Thus, transparent objects such as certain insect larvae are very suitable for phase-contrast microscopy, even though their thickness is appreciable (Barer⁶). Thin, single cells such as certain

protozoa, spermatozoa, and cells in tissue culture are ideal objects. Cells from almost any tissue can be examined in the teased state. Such cells may be flattened slightly by mounting under a cover-slip, but any greater degree of flattening is strongly to be deprecated. Many observations have shown that such treatment causes pronounced changes in the structure of the cell, which can no longer be regarded as normal or even viable.

Phase-contrast microscopy gives the cytologist what he has often sought but never hitherto attained—a method of observing *living* cells under excellent optical conditions. It is at last possible to be reasonably certain that what is seen bears a good resemblance to what is actually present in the living cell. The usual method of examining such cells in the past has been to reduce the condenser aperture while altering the focus of the objective. It is not generally appreciated that under these conditions the image is best seen only when it is slightly out of focus. This is, in fact, a very crude method of obtaining phase-contrast, by introducing optical path differences between rays of different obliquity, due to change of focus. At the same time, the reduction of the condenser aperture results in a serious decrease in resolving power, and detail tends to be lost in a haze of diffraction rings. The phase-contrast films of mitosis in tissue culture cells made by Michel in Germany and Hughes at Cambridge illustrate the great technical superiority of phase-contrast over the older methods. From the point of view of cytological research phase-contrast enables us to study such things as the chromosomes, mitochondria, and Golgi apparatus in the living state, free from artefacts due to fixation and staining. Some reorientation of our views on cell structure is clearly necessary. Whereas in the past our conception of the 'normal' cell has been based on the appearance following some particular method of fixation and staining, we must now take the living cell as our standard and see how far its appearance has been modified by routine histological procedures. Phase-contrast microscopy reveals the presence of numerous cytoplasmic inclusions in the living cell. These can generally be divided into two broad classes, small dark (by positive phase-contrast) granules and rodlets, generally numerous, and larger bright spherical bodies or vacuoles with dark rims, generally only few in number. The dark bodies seem to correspond to the classical description of the mitochondria, while some of the larger spherical bodies resemble the Golgi apparatus as described by some authors. Crawford⁴¹ has studied the action of vital dyes on these inclusions and has found that the dark granules stain with Janus dyes, whereas the spherical bodies take up Neutral Red. In most cases Neutral Red appears to accumulate in preformed granules or vacuoles, but this is not always so. It is not proposed to discuss here the relation of these results to the structure of the Golgi apparatus, but it should perhaps be stated



that in the course of a study of living cells taken from very diverse material nothing closely resembling the classical Golgi 'network' has been seen (Barer and Crawford, unpublished observations; see also Brice *et al.*²⁵, and Oettlé⁸³).

It is clearly a matter of some importance to determine the structural alterations produced by the action of fixatives and other reagents on living cells. In the past this problem has been investigated by immersion of pieces of tissue in a fixative, followed by clearing, embedding, sectioning, and staining (Hardy⁵⁸). Strangeways and Canti¹⁰⁹ observed the action of fixatives on living tissue culture cells by dark-ground illumination. This eliminated changes produced by the histological procedures subsequent to fixation, as well as artefacts due to staining, but the observations were limited by the defects inherent in dark-ground illumination. An extensive survey of the action of fixatives and other chemical and physical reagents on living cells and protein films is now being undertaken, using phase-contrast microscopy (Barer and Crawford¹¹). The results will be described in detail elsewhere, but it has already been observed that the action of several common fixatives on isolated cells is very different from the classical description. There is no doubt that phase-contrast microscopy has an enormous scope in investigations of this sort, in all types of material. Thus, it is possible to observe the action of drugs and antibiotics on living bacteria. Streptomycin has been found to produce several types of abnormal forms, such as giant filaments or 'snakes', and bulbous forms. Various types of granules can be seen in some living bacteria, and the question arises as to whether these may be some form of nuclear apparatus, as has been described in stained preparations by Robinow⁹⁸. No definite answer can be given until the changes undergone by granules during bacterial growth cycle have been observed. Nor can the granules be identified with those seen by Robinow at this stage. Preliminary observations indicate that the fixatives and acid hydrolysis used in the staining procedure may themselves produce dense 'bodies' within the bacteria (Barer and Barer¹⁰).

Apart from observations on isolated cells, phase-contrast microscopy can be used for the study of suitable material in intact animals and tissues. The study of whole insect larvae has already been mentioned. Transparent tissues such as the mesentery of mammals and amphibia, and the tails of tadpoles (particularly *Xenopus* tadpoles) also provide interesting material for study. The use of thin transparent chambers inserted in the ears of rabbits should also prove valuable provided that certain technical difficulties can be overcome.

Micro-dissection by Phase-Contrast Illumination. Micrurgical methods as developed by Chambers^{35,36} and others have given us valuable insight into the physical properties of cells, despite the limitations imposed by the use of unsatisfactory methods of microscopical observation. With

the advent of phase-contrast microscopy we may look forward to a considerable extension and improvement of these methods. The ordinary commercial phase-contrast equipment is not suitable for micro-dissection work at high magnifications, but through the kind co-operation of Messrs. Cooke, Troughton, and Simms the writer has been able to obtain a specially designed condenser with a long working distance, adequate for work with cells suspended in hanging drops in a moist chamber. The technique of micro-dissection by phase-contrast illumination is by no means easy. The optical conditions are liable to be disturbed by the introduction of micro-instruments between the condenser and the object, and by the presence of a convex hanging drop, which may act as a lens. It is generally better to use a thin film of liquid for suspension of the cells, rather than a drop. A microscope with a focusing stage as well as a focusing body is also desirable. An inverted microscope would probably be even better.

Resolving Power. It is frequently asked how the resolving power of phase-contrast illumination compares with that of conventional dark-ground illumination. In theory, the resolving power depends on the NA of that part of the objective aperture which is being illuminated, i.e. the NA of the annulus of the phase plate. In most cases this is about two-thirds to three-quarters that of the full-rated NA of the objective, though some equipment uses the full NA. It must be remembered that the full NA of an objective is rarely used in conventional microscopy, owing to glare, and a $\frac{3}{4}$ -cone of illumination is usually regarded as quite satisfactory. We should thus expect to find that the resolving power of phase-contrast and conventional illumination is in practice roughly equal. The overriding consideration in the case of living cells is contrast, rather than resolution, for there is no point in discussing the resolution of invisible objects! It would be better to sacrifice resolution to some extent than to see nothing at all. However, in practice, the phase-contrast method appears to provide a resolving power at least as good as that of an objective of equivalent NA used with conventional illumination. It is even possible to see particles below the theoretical limit of resolution, provided the contrast is sufficiently high. This is, of course, well known in dark-ground illumination, in which the ability to detect a particle is largely a matter of using a sufficiently intense source of light. In (positive) phase-contrast, particles below the limit of resolution appear in progressively lighter shades of grey, as their size diminishes. It is possible to resolve some of the larger viruses, and to detect the presence of even smaller particles by phase-contrast microscopy (Barer⁹). The dark-ground method may be more sensitive for detection of small particles, but it must be remembered that it does not give such a true representation of larger objects. Phase-contrast microscopy is vastly superior in this respect.

The Possibility of Optical Artefacts. It is curious with what suspicion phase-contrast microscopy was, and often still is, regarded by many microscopists. It is true that there are many theoretical and practical points which are not fully understood, but the same can be said of conventional microscopy, and dark-ground illumination. One so often hears the remark 'Couldn't it be a diffraction effect?' that it is difficult to resist replying 'Yes, a *double* diffraction effect!' What the practical microscopist wishes to know is how far he is justified in regarding the image he sees as an exact representation of the object. No straightforward reply can be given to this question for *any* method of microscopy, but we can assert with reasonable confidence that the image of a transparent object when viewed by phase-contrast illumination is neither more nor less reliable than is the image of a stained object when viewed by conventional illumination. As a general rule the possibility of gross optical artefacts can be ruled out. Difficulties in interpretation may arise, particularly when the object contains a mixture of transparent and absorbing detail, and it may be necessary to examine the appearance and behaviour of bodies of known structure. Thus, it is often useful to compare the appearance of a body seen by phase-contrast, just above and just below focus, with that of a known absorbing body such as a carbon particle when using conventional *annular* illumination and passing above and below focus.

One effect which is inherent in the phase-contrast method is the appearance of a bright (in positive phase-contrast) halo around the object. This is due to the way in which energy is redistributed by the phase plate. It will be appreciated that if by phase-contrast a transparent object is rendered black or in shades of grey, the light energy has not been absorbed, as is the case with a stained object, but has merely been redistributed. This redistribution manifests itself as a bright halo around the object. With a properly designed system this effect should not be obtrusive.

c. Interference Microscopy

Interferometric methods are commonly used in physics for the determination of refractive indices of liquids and gases. This is done by observing the shift of a fringe system when the introduction of a specimen causes the optical path to vary. It is obvious that the principle might be extended to microscopical objects such as living cells, and would perhaps yield valuable information concerning variations of refractive index and thickness in the cell. There has been a considerable revival of interest in such methods. One of the simplest, originally described by Frederikse⁴⁸ and considerably improved by Merton^{77,78}, depends on observing the specimen between two thin semi-reflecting plates (see also Ambrose³). Under these conditions a system of

interference fringes is produced by multiple reflections between the plates. The specimen introduces variable optical path differences, which is equivalent to varying the separation of the two plates, with consequent disturbance of the fringe pattern. Thus some parts of the specimen are made to appear bright against a dark background, and vice versa. Interesting colour effects may also be observed. These changes of intensity and colour result in increased contrast, and indeed the appearance of a living cell is very like that seen by phase-contrast illumination. There are technical difficulties which limit the resolving power of the method, and it is necessary to use thin specimens. Nevertheless, the most recent modifications of the method, involving the use of zone plates to ensure more even illumination of the objective, are distinctly promising. The writer has had the opportunity of observing the results obtained with the latest developments, through the kindness of Dr. A. C. Menzies, and it certainly appears that the method is worthy of serious consideration.

Many other varieties of interference microscope are possible, some of them being exceedingly complicated. The more ambitious forms would require as many as four objectives! Dr. J. St. L. Philpott has obtained interesting results using vertical illumination through a specially constructed double prism, the object being mounted between a plane mirror and a cover-slip. The interference fringes are produced by quite a different mechanism from that described above, but the appearance of the cell is somewhat similar.

Some extremely ingenious experiments have been carried out by Mr. W. J. Bates, working in Dr. Burch's laboratory. By an adaptation of his wavefront-shearing interferometer (Bates²⁰) he has been able to obtain a *double* image of a cell, one in bright contrast, the other in dark contrast. Other developments of the method include the possibility, already referred to, of obtaining the equivalent of a phase-contrast effect with complete control at will of both phase and amplitude characteristics.

There seems little doubt that interference microscopy is assured of an important future, not perhaps as a routine weapon, but as a useful accessory to other methods, particularly when rather different types of contrast are desired.

d. Fluorescence Microscopy

It is well known that when some substances are illuminated by light of a certain wave-length energy is absorbed and then partly re-emitted as light of a longer wave-length. This phenomenon is known as fluorescence, and it provides a useful method of obtaining contrast in microscopy. In order that the fluorescent image may be of a colour visible to the eye, the exciting illumination is generally ultraviolet light. For most work it has been found that the range of wave-lengths between 300 and 400 m μ is most convenient and effective. In passing it should

be noted that fluorescence microscopy differs in one important respect from most other methods of microscopy, for we are here dealing with a *self-luminous object*, whereas in most other cases the object is illuminated by 'borrowed' light.

Apparatus. The essential requirements are (1) a source of ultraviolet light. The most convenient source is undoubtedly some form of mercury arc such as the high or medium pressure arcs sold by several makers (G.E.C., B.T.H., Siemens, &c.). Carbon arcs and spark discharges may also be used. (2) A method of eliminating all but the desired excitatory rays. In particular, excess visible light must be removed since the fluorescent image is usually very faint and may be masked. This is particularly important for photography. For most work a filter of the Wood's glass type, passing wave-lengths between 320 and 400 m μ , is quite satisfactory. If desired, red and infra-red rays may be removed by a 20 per cent. copper sulphate solution. Other filters may also be used (see Bowen²⁴). Monochromators are not generally employed since the intensity of illumination is usually too low. (3) The direct ultraviolet radiation must be excluded from the eye or photographic plate. Special cover glasses have been manufactured for this purpose, but a suitable filter may be used below or above the eyepiece.

Certain features are desirable in the microscope. It is frequently stated that a quartz condenser is desirable or even essential. However, most glasses will transmit wave-lengths above 320 m μ , and if a Wood's glass filter is used there seems very little point in having a condenser transparent to lower wave-lengths. For special research purposes quartz condensers or reflecting condensers may be useful. A front-surface aluminized mirror or a quartz 90° reflecting prism are valuable accessories. The usual back-surface silvered mirror is inefficient as silver shows a sharp minimum in reflectivity below 350 m μ . It is most important that with the exception of the specimen itself the light path should be quite free from fluorescent materials. Some glasses are fluorescent and special care should be taken to test slides, cover-glasses, lenses, and eyepieces. The use of objectives containing fluorite must be avoided. Many mounting media and immersion oils are also fluorescent (e.g. Canada balsam and cedar wood oil). Glycerine and water can be used freely.

Two types of fluorescence may be recognized.

1. Primary fluorescence, which is exhibited by the object in its natural state.
2. Secondary fluorescence, exhibited by the object after impregnation with fluorescent dyes (fluorochromes).

Primary fluorescence is clearly of much greater fundamental importance since it is an indication of a definite chemical property of the object itself. The use of fluorescent dyes to increase contrast is probably no more specific than the use of routine staining methods in conventional

microscopy. Nevertheless, it may prove useful in certain problems. The comprehensive review by Ellinger⁴⁶ should be consulted for a summary of the results obtained by the use of fluorescent dyes. Ellinger and his collaborators have carried out extensive investigations on the circulation in various organs illuminated by incident light, using fluorescein. The functions of the liver and kidney have been investigated in this way. Other noteworthy examples of the use of fluorescent dyes are the selective staining of tubercle bacilli—a valuable routine laboratory method, and of certain viruses (Hagemann^{52,53}). Primary fluorescence has proved useful in the investigation of such substances as porphyrins and some vitamins. Dempsey⁴³ has investigated the action of such drugs as thiouracil and thyroxine on the fluorescence of the colloid substance in the follicles of the thyroid gland. Many synthetic carcinogenic compounds are strongly fluorescent, and it has been claimed that they can be located within tissues and cells by fluorescence microscopy (Simpson and Cramer¹⁰⁶; Doniach, Mottram, and Weigert⁴⁵; Ahlström and Berg²). There is no doubt that a great deal remains to be done in the investigation of primary fluorescence. The ability to use high magnifications depends on the use of an intense source and an efficient condenser. Barnard and Welch^{16,18} have employed spark sources and a special dark-ground reflecting condenser designed by Smiles¹⁰⁷. The use of dark-ground illumination helps to ensure that no direct ultraviolet light enters the objective, but it should be emphasized that the image seen is a bright one due to the self-luminous (fluorescent) object. The remarkable photographs published by Barnard and Welch¹⁶ showing the same bacteria by ultraviolet and fluorescence microscopy represent the highest performance hitherto attained by fluorescence microscopy. Further investigations using both dark-ground condensers and achromatic reflecting condensers of the Burch type would be very valuable.

e. *The Polarizing Microscope*

Like fluorescence microscopy, polarizing microscopy is a special method for obtaining contrast and at the same time is capable of yielding valuable physico-chemical information in certain cases.

The majority of crystalline substances are *anisotropic*, i.e. they exhibit different physical and optical properties in different directions. Substances in which the refractive index (and therefore the velocity of light) varies with direction are known as *doubly refracting* or *birefringent*. Substances in which the absorption of light varies with direction are called *pleochroic*.

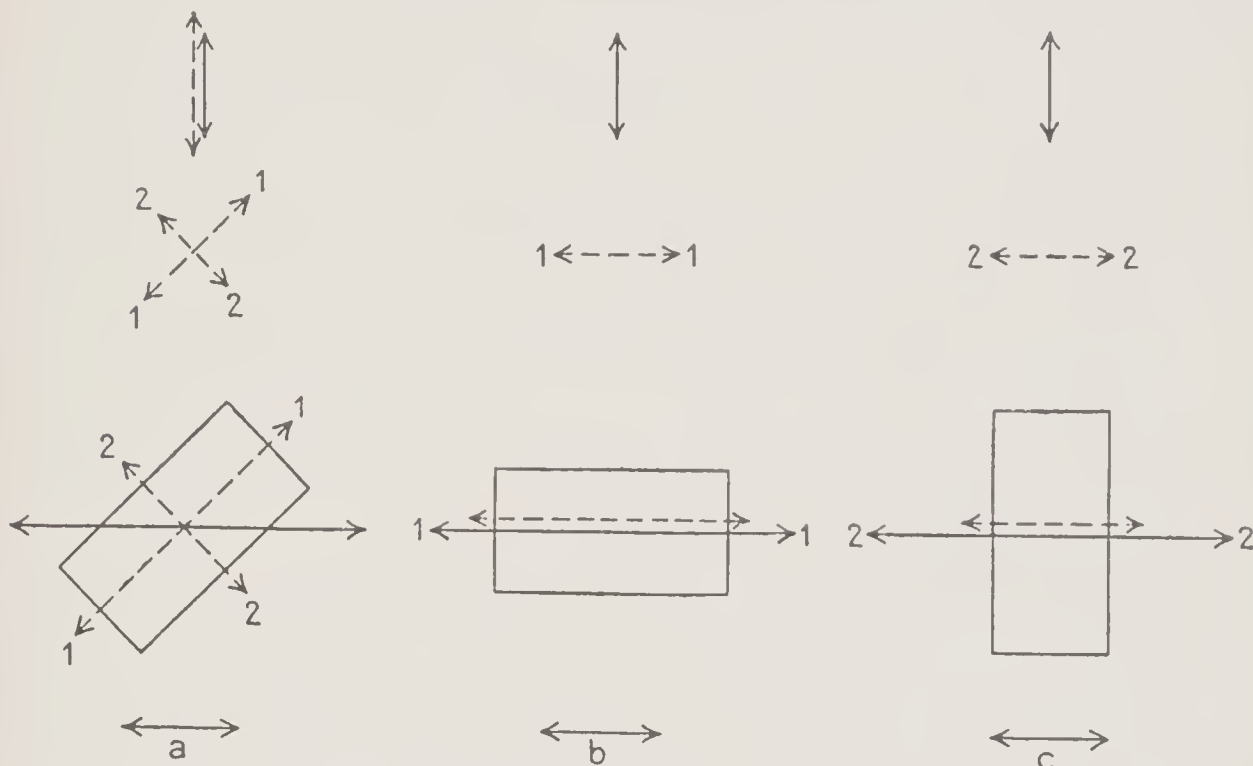
A beam of light consists of electromagnetic waves vibrating in all possible planes. On passing through an anisotropic substance these irregular vibrations are resolved into components which can only vibrate

in two fixed perpendicular planes. The light is said to be *polarized* in these two planes. The velocity of the two polarized beams differs so that they undergo unequal refraction in passing through the birefringent substance. If the substance is also pleochroic, the intensity of the two beams will be affected unequally. In order to obtain a single beam of plane polarized light it is necessary to remove one of the two polarized beams. In the case of the Nicol prism this is accomplished by total reflection of the unwanted beam at a layer of Canada balsam. Another very convenient method is to utilize crystals with a high degree of pleochroism. It has long been known that crystals of iodo-quinine sulphate (Herapathite) exhibit this property to a remarkable extent, so that one of the two polarized beams is completely absorbed in passing through a very thin layer of the crystal. Such crystals are used in different ways in Polaroid, the Marks plates, and the Zeiss Bernauer filters. Recently some very simple polarizing films have been made by orienting dye molecules in stretched plastic sheets. The great value of such polarizing plates is that they can be obtained in very large sizes and are relatively cheap as compared with Nicol prisms. It is clear that if a beam of plane polarized light is made to fall on an anisotropic body it will in general itself be resolved into two mutually perpendicular plane polarized beams. However, if this anisotropic body is itself a polarizer, i.e. a Nicol prism or a Polaroid plate, which eliminates one of the polarized beams, and if the plane of the incident polarized beam is perpendicular to that which the second polarizer allows to pass, complete extinction of the incident beam will result. Two polarizers arranged in such a manner constitute a polarizer and analyzer system. The intensity of the light passed by such a system should theoretically vary from zero to a maximum as the planes of polarization of the polarizer and analyzer are varied from perpendicular (crossed) to parallel.

Let us now consider the effect of introducing a birefringent body between a 'crossed' polarizer and analyzer. The body will be illuminated by a beam of plane polarized light which it will resolve into two plane polarized beams. These two beams will pass through the body with different velocities and will therefore arrive at the analyzer *out of phase*. For the sake of simplicity let us assume we are dealing with a rectangular birefringent object, which polarizes light in two directions parallel to its sides. In Text-fig. 6 (a) it will be seen that if neither of these directions is parallel to the plane of the incident polarized beam the latter will be resolved into two components in the directions 11, 22.

In Text-fig. 6 (b) and (c) one of the directions of polarization is parallel to the plane of the incident beam. In this case *all* the incident light will continue to vibrate in the same plane and there will be *no* component in any other direction. On arrival at the analyzer, in the case shown in Text-fig. 6 (b) and (c), none of the light which has passed through

the birefringent object will pass through the analyzer since it is vibrating in a direction at right angles to that which the latter will pass. In Text-fig. 6 (a), however, each of the components in the direction 11 and 22 will in turn be broken up into components parallel and perpendicular to the permitted direction of the analyzer. The components



TEXT-FIG. 6. Diagram illustrating the action of a birefringent object on a beam of plane polarized light (see text).

parallel to this direction will pass through so that the object will appear bright against a dark field. If the object is rotated through 360° it will pass from darkness through maximum brightness and back to darkness in every quadrant. As we have already seen, the matter is further complicated by the fact that in Text-fig. 6 (a) the two beams in the directions 11 and 22 are out of phase on arrival at the analyzer. They are thus in a position to interfere. Since the phase difference depends on wavelength, different colours are affected unequally so that brilliant interference colours may be produced. It is possible to measure the degree of birefringence of an object quantitatively by using a *compensator*. This consists of a birefringent plate which produces a known phase difference (or retardation) between the polarized beams it transmits. If this compensator is introduced between the polarizer and analyzer it superimposes a known phase difference on that produced by the object. The contrast of the latter can thus be neutralized or enhanced by adjustment of the compensator.

Apparatus. Any microscope can be converted into a simple polarizing microscope by fitting a polarizer in the substage and an analyzer somewhere above the objective (just above or below the eyepiece is

usually the most convenient place). With the advent of polaroid there is no reason why simple examinations in polarized light should not be carried out by all microscopists using standard microscopes.

For research purposes, particularly where quantitative measurements are required, the standard type of biological microscope is quite unsuitable. The minimum requirements for this type of instrument are:

1. A polarizer and analyzer, both capable of accurate rotation, the rotation of one being readable by a vernier to a fraction of a degree.
2. A series of interchangeable compensators which can be inserted into the body of a microscope between the objective and eyepiece. These compensators should be capable of accurate adjustment, and should measure retardations down to the order of one-tenth of a wave-length or less.
3. The microscope should be equipped with a good rotating and centring object stage. The condensers and objectives should be specially chosen to be free from birefringence.

Such microscopes, suitable for detecting and measuring very weak birefringence in biological objects have hitherto only been obtainable on the continent (notably Leitz and Co.). Recently an improved polarizing microscope for petrological work has been produced (Hallimond and Taylor^{55,56}), and modifications of this instrument have proved quite suitable for biological work. An interesting feature is the use of Polaroid instead of polarizing prisms. This would have been unsatisfactory with early samples of Polaroid which rarely gave complete extinction when crossed, but selected pieces of the newer material give a greatly improved performance and have not been found inferior to Nicol prisms (Hallimond⁵⁴). The use of Polaroid confers certain important advantages. Owing to the expense and difficulty of obtaining large Nicol prisms it was necessary to use specially constructed small substage condensers. It is now possible to use any standard condenser. Another disadvantage of the prism is that it introduces astigmatism, which requires at least two extra correcting lenses for its elimination. The introduction of extra reflecting surfaces inevitably leads to a loss of contrast. The best type of compensators for exacting biological work are the rotating plates described by Köhler⁶⁹. These can be obtained for measuring retardations lower than one-twentieth of a wave-length. Even weaker degrees of birefringence have been measured and by attention to details of design it should be possible to increase the sensitivity of the method very considerably. Valuable work in this direction is now being carried out at Cambridge by Swann and his collaborators. By using front-surface mirrors, double-thickness Polaroid, bloomed lenses, and hand-made compensators, retardations of the order of one

fifteen-hundredth of a wave-length have been measured (Hughes and Swann⁶²).

The writer has found it very useful to be able to examine the same object by phase contrast and by polarization microscopy. He uses a polarizing microscope of the type described above, equipped with phase-contrast condensers and objectives selected for low birefringence, enabling a rapid changeover from one type of illumination to the other to be carried out. This is particularly valuable for the study of unstained material and living cells.

Results. Only the main types of information obtained by polarization microscopy will be indicated here. For details the reviews of Schmidt¹⁰¹, Schmitt¹⁰², Picken⁹⁰, and Frey-Wyssling⁴⁹ should be consulted. Birefringence in biological objects may be due to a regularity of molecular structure (intrinsic or crystalline birefringence) or to an orientation of asymmetric particles or micelles (form birefringence). Some materials exhibit birefringence only when subjected to mechanical stress (strain birefringence). Intrinsic birefringence can be distinguished from form birefringence by immersion of the object in fluids of different refractive indices. Form birefringence is completely neutralized by immersion in a fluid of the same refractive index as that of the micelles, whereas intrinsic birefringence is unaffected. Measurements of the way in which birefringence varies with the refractive index of the surrounding medium enable us to estimate the degree of both intrinsic and form birefringence, and it is also possible to calculate the volume occupied by the orientated micelles. The ultra-structure of muscle (Fischer⁴⁷; Buchthal and Knappeis²⁹; Schmidt¹⁰⁰) and nerve (Schmitt and Bear¹⁰³; Richards⁹⁵; Pfeiffer⁸⁸) have been investigated in some detail using such methods. Interpretable quantitative results are less easy to obtain on non-fibrous cells, but birefringence has been detected in numerous cell structures. Pfeiffer⁸⁷ combined a sensitive polarizing microscope with the Harvey-Loomis centrifuge microscope, and was able to observe the development of birefringent regions in the frog ovum undergoing centrifugation. Moore and Miller⁸⁰ examined egg cells after centrifugation and found that the cytoplasm of fertilized eggs showed birefringent areas. Monné⁷⁹ has made similar observations. These results may very well indicate the existence of a cellular organization similar to the 'cytoskeleton' suggested by Peters^{85,86}. Other intracellular structures whose birefringence has been studied are the chromosomes, asters, and spindles. An interesting study of the cytoplasm, cell membrane, and nuclear membrane of nerve cells has been carried out by Chinn³⁷. It was found that the protein micelles in the cytoplasm of unipolar and multipolar nerve cells are orientated in different directions. The nuclear membrane shows weak birefringence due mainly to orientated lamellar protein micelles (see also Pfeiffer⁸⁹). It should be mentioned that the birefringence due to one

substance or structure can be masked by an opposite birefringence due to another substance. We have seen that the two rays which arrive at the analyzer are out of phase, i.e. one arrives a little before the other. It may happen that this state of affairs is neutralized or reversed by the presence of some substance which affects the two rays in an opposite fashion. Without going into the optical details it can be said that in biological systems the proteins and the lipoids quite often produce antagonistic types of birefringence. Thus, the myelin nerve sheath shows what is known as positive birefringence. On extraction of the lipoids only a weak negative birefringence, due to the proteins, can be detected. The possibility of this type of masking must constantly be borne in mind. According to Dempsey, Wislocki, and Singer⁴⁴ the I (isotropic) disk of the striated muscle fibre is in fact birefringent, provided that lipoids are extracted. In the past numerous erroneous conclusions were drawn as to the structure of the muscle fibre due to ignorance of such effects.

The analysis of birefringence can at best yield indirect information. It cannot be regarded as a conclusive or reliable method when taken by itself, but in conjunction with other methods such as X-ray diffraction and electron microscopy it provides an essential link in the chain of circumstantial evidence upon which our conception of cell structure is based.

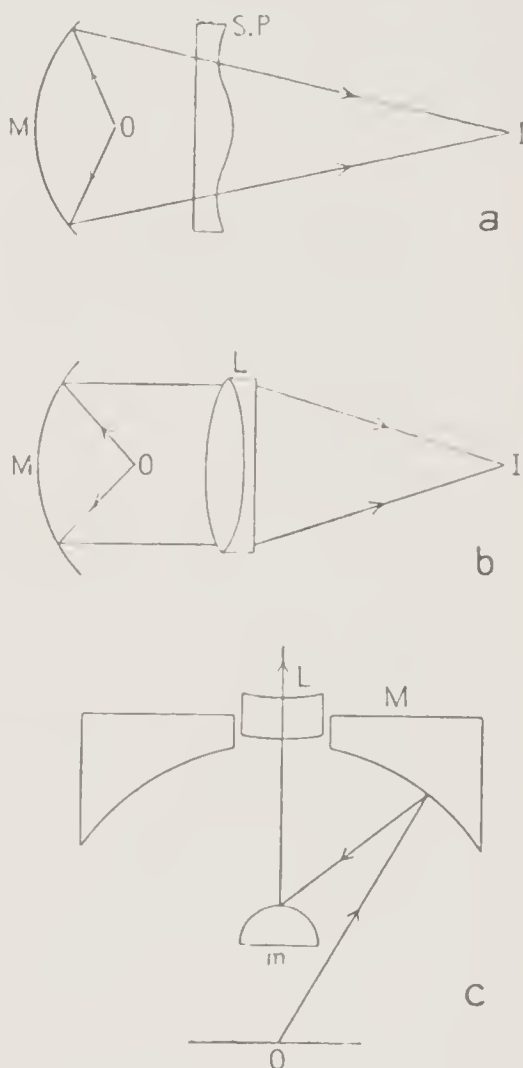
f. The Reflecting Microscope

One of the most important objects in optical design is the reduction or elimination of chromatic aberration. The introduction of the achromatic microscope objective lens (Lister, 1829; Amici, 1827) marked the beginning of a new era in microscopy. The introduction of the apochromat by Abbe was an equally great advance. It must be emphasized, however, that the terms 'achromatic' and 'apochromatic' are somewhat misleading. The ordinary microscope objective lens is very far from being truly achromatic—it is merely the best that the lens maker can do with the materials at his disposal (Text-fig. 2). True achromatism can only be achieved by a reflecting system. It is curious that although reflecting telescopes have been in use for many years, relatively few attempts have been made to construct high-performance reflecting microscopes. No less an optician than Newton conceived a simple reflecting microscope and from time to time others have appeared (two such instruments are in the Museum of the History of Science at Oxford, one by Amici, the other by Cuthbert).

It was soon apparent that although such instruments gave reasonably good performances at low magnifications, their use was limited by their low NA (less than 0.1). Attempts to increase the NA were accompanied by considerable distortion in the image. In recent times several workers

have renewed the attack on the problem. In Russia, Maksutov⁷⁴ pointed out that a combination of a spherical and a cardioid mirror (as used in some dark-ground condensers) would act as a satisfactory microscope objective. Brumberg²⁷ has described an objective composed of two spherical mirrors, one concave, the other convex, together with a lens to correct for the chromatic aberration of the cover-glass (Text-fig. 7). This type of objective can be used with NA up to about 0.5, but above this spherical aberration becomes very serious. A disadvantage of this particular design is that a relatively large fraction of the aperture of the concave mirror is obstructed by the shadow of the small convex mirror. Other simple reflecting microscopes of low NA have been constructed in Holland (see Bouwers²³).

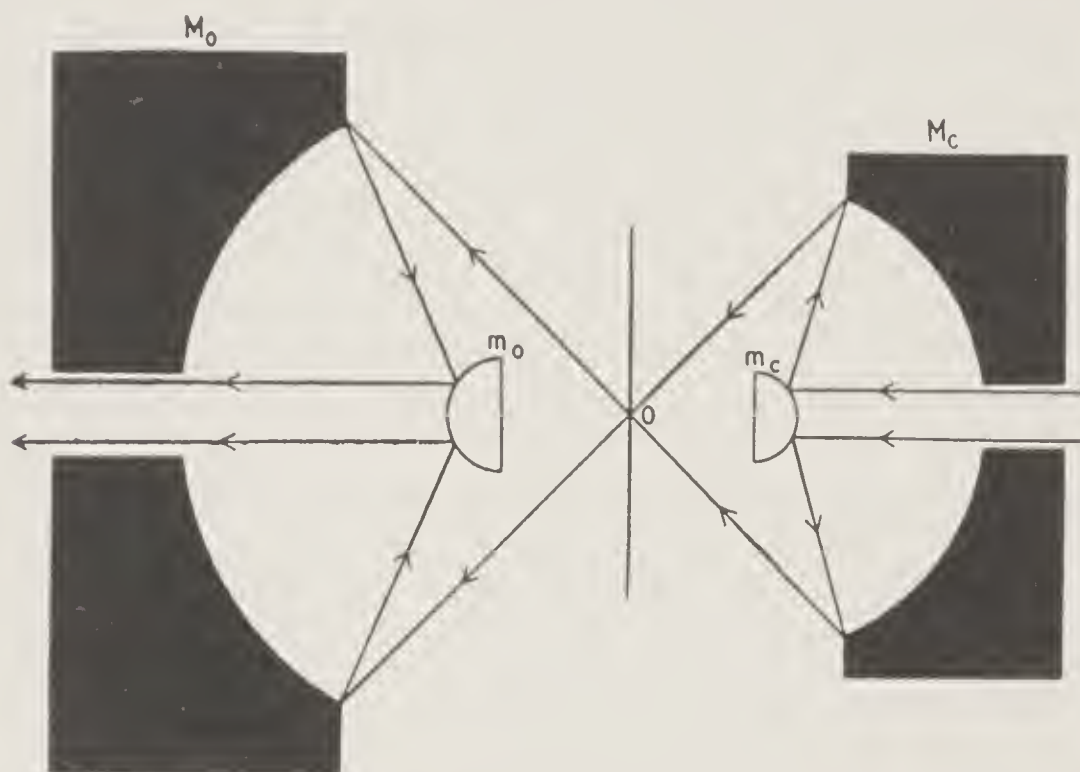
Several microscopes combining both reflection and refraction have been described. Linfoot⁷¹ used a microscope built on the Schmidt principle. He used a quartz aplanatizing plate (Text-fig. 7) and obtained good results with an NA of 0.54. Johnson⁶⁵ combined an 'achromatic' lithium-fluoride-silica lens with a parabolic mirror (Text-fig. 7). The system was computed to bring wave-lengths 5,416 Å and 2,749 Å to the same focus, and it was found that the corrections were reasonably good for all wave-lengths between 6,000 Å and 2,600 Å. An NA of 0.5 was achieved. While there is no doubt that further advances may be expected along these lines, it seems probable that a purely reflecting system offers the best approach to the problem of achromatization. The chief difficulty is that an NA above 0.5 cannot easily be obtained without the introduction of considerable spherical aberration. In 1905 Schwarzschild investigated the theory of the construction of a two-mirror telescope objective. It was realized by Burch³⁰ that this theory could be very easily modified to apply to a two-mirror microscope. He showed that in order to minimize spherical aberration at high NA, at least one of the two mirrors has to be aspherical. For an NA up to about 0.65 it is possible to use a large concave aspherical (approximately ellipsoidal) mirror in combination with a small convex spherical mirror.



TEXT-FIG. 7. Three types of reflecting microscope.

- (a) Schmidt system (Linfoot), with spherical mirror *M* and correcting plate *S.P.*
- (b) Parabolic mirror *M* and achromatic lithium-fluoride-silica lens *L* (Johnson).
- (c) Small convex (*m*) and large concave (*M*) mirrors, with correcting lens *L* (Brumberg).

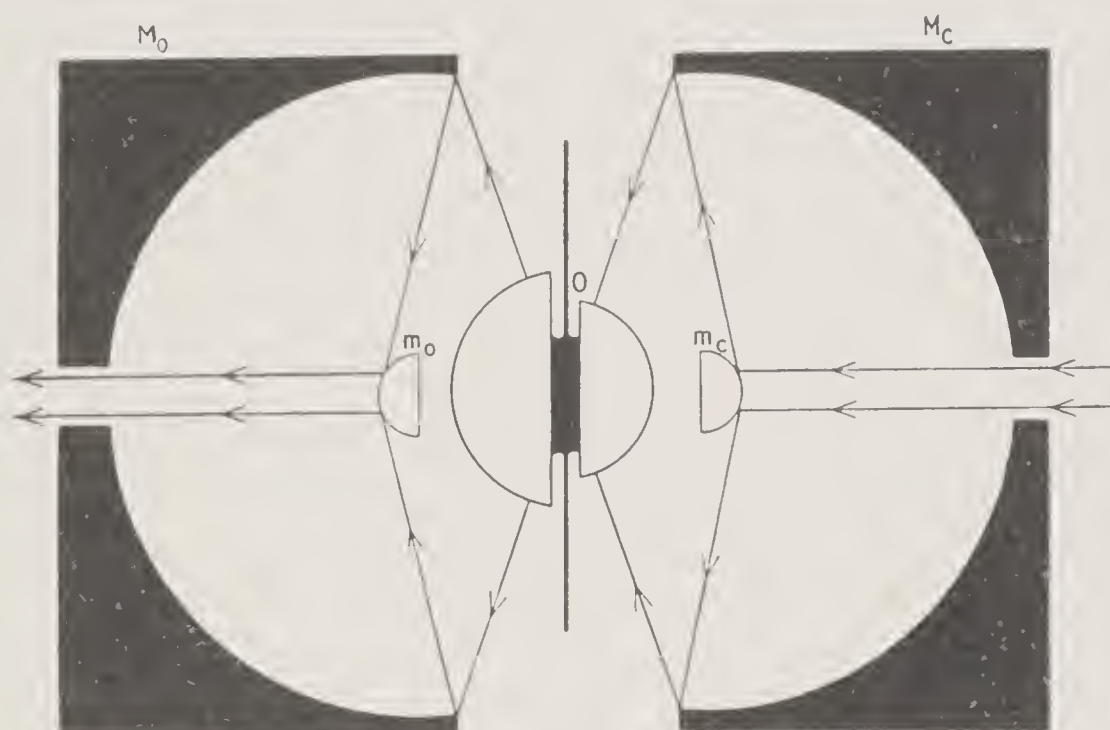
For larger NA both mirrors must be aspherized. The convex mirror can be made small enough not to obstruct any significant part of the aperture of the large mirror. Text-fig. 8 shows in its simplest form the optical system of the Burch microscope in use by the writer at Oxford. A reflecting objective (NA 0.65) and reflecting condenser (NA 0.58) are used. The mirrors are of speculum metal, the final polishing being done by



TEXT-FIG. 8. Burch system without immersion lenses.
NA 0.65 (0.98 with immersion lenses). M_o , M_c are aspherical, m_o , m_c are spherical.

hand, the exact shape being controlled by means of an interferometer system. It will be appreciated that considerable labour and skill are required. The spherical aberration of the system is very easily corrected by altering the distance between the large and small mirrors. It is thus possible to minimize the aberration introduced by a cover-slip. Dr. Burch and his collaborators are at present engaged on the construction of a reflecting microscope having the highest possible NA. As we have already seen no 'dry' system can have an NA greater than 1.0. The projected microscope will have an NA of approximately 0.95. Both mirrors will be aspherical, and the large mirror will be greater than a hemisphere. It is possible to increase the NA of the present instrument to 0.98 by means of an immersion-component lens. At first sight this might be expected to upset the achromatism of the system, but if the lens is made with a spherical surface, with its centre at the object point, rays will pass through radially so that the achromatism of focus is unaffected. Chromatic error of magnification is introduced but this is unobtrusive in practice and could be eliminated if desired by the use of an auxiliary lens. The NA of the projected instrument will be increased to 1.4 by the use of similar immersion components (Text-fig. 9).

The performance of the reflecting microscope in the visual range is as good as, if not better than, that of the best apochromatic lens microscopes of equivalent NA, and it has the additional advantage of an unusually flat field of view. Photomicrographs taken with the instrument have been published elsewhere (Burch³⁰, Barer⁷). The microscope has been used with excellent results with phase-contrast illumination, and for polarizing and fluorescence microscopy.



TEXT-FIG. 9. Burch system, now under construction, showing immersion lenses on condenser and objective.

NA 0.95 dry, 1.4 with immersion components. All mirrors aspherical.

APPLICATIONS

A brief consideration of some of the possible applications of the reflecting microscope suffices to make one realize that this is one of the most powerful cytological weapons ever constructed. It is *not* merely an improvement on existing refracting microscopes. It opens up new fields which it would be quite impossible to explore by present methods.

Achromatism

In the first place the implications of true achromatism are often not fully understood. A reflecting system is achromatic not merely for the *visible* range, but for *all wave-lengths from the far ultraviolet to the far infra-red*. Ultraviolet microscopy (Köhler⁶⁸; Barnard¹⁴; Lucas⁷²; Wyckoff¹¹⁵) and particularly ultraviolet absorption microspectroscopy, as developed by Caspersson and others, has made contributions of fundamental importance to cytochemistry. The technique employed has differed little since the time of Köhler⁶⁸, and is generally somewhat cumbersome and unsatisfactory. Since glass will not generally transmit wave-lengths below 3,200 Å.U. it is necessary to employ quartz lenses.

Theoretically it is possible to make an 'achromatic' lens of quartz and fluorite, but this is exceedingly difficult in practice and such lenses have rarely been used. The use of a single material (quartz) means that the objective can only be used with strictly monochromatic light. Most quartz monochromats are corrected for minimum spherical aberration at 2,750 Å.U. It is possible to use them within a narrow range of wave-lengths on either side of this, but there is always some deterioration of the image. Perhaps the most serious difficulty in ultraviolet (and infra-red) microscopy is that since the image is invisible there is no satisfactory means of focusing. A fluorescent eyepiece can be used, but in general focusing becomes a matter of trial and error. Elaborate mechanical focusing devices involving the use of interchangeable eyepieces have been employed but even these require empirical calibration. For a full account of standard quartz microscopy the reader is referred to the papers of the workers at the National Institute of Medical Research, Hampstead (Barnard, Welch, Smiles^{14,15,17,107,108}). In the opinion of the writer the quality of their photographs has never been excelled.

The use of a truly achromatic system eliminates difficulties in focusing. The object can be focused in visible light of any colour and a photograph taken in any wave-length without change of focus. It is not even necessary to use monochromatic light. (These statements are not strictly true when a normal cover-slip is employed without an immersion component, since the dispersion of the cover-slip material would introduce chromatic error.) All that is required is that the system should pass sufficient light. Speculum metal is a poor reflector of ultraviolet light, so for work in this range the mirrors are aluminized by evaporation *in vacuo*. Quartz or fluorite immersion components are needed, but their construction is very simple. The use of a fluorite immersion component should enable the instrument to be used at wave-lengths down to about 1,200 Å.U. No material sufficiently transparent to lower wave-lengths and suitable for making lenses is known, so that the instrument would have to be used 'dry' at shorter wave-lengths. The value of ultraviolet microscopy is twofold. In the first place, as we have already seen, the resolving power of a microscope is inversely proportional to the wave-length. Thus for the same NA the resolving power (and useful magnification) at 2,750 Å.U. should be about double that for green light. Secondly, some cellular constituents show strong absorption at certain wave-lengths. Thus, the nuclei of cells absorb strongly at about 2,600 Å.U. The result is that a photograph of a living cell taken at this wave-length will show a dark nucleus, as if the cell had been stained. The use of shorter wave-lengths would confer the advantages of greater resolving power. Thus, at 1,200 Å.U. the resolution should be about four times as good as in the visible range—a considerable improvement. There seems no reason to doubt that such wave-lengths will be

attainable. It is true that the microscope will have to be used in a vacuum chamber or at least in a special gas chamber since the oxygen in the air absorbs strongly below 1,800 Å.U., but this is not a serious obstacle. The employment of even shorter wave-lengths would be a more difficult procedure. It must be pointed out that the mirror surfaces have to be figured correctly to a fraction of a wave-length. Naturally, the shorter the wave-length the greater the accuracy required. It remains to be seen how accurately such surfaces can be made. Another serious difficulty is the fact that the reflectivity of most substances appears to fall rapidly below 1,000 Å.U. Much more research into this aspect of the problem is required. For the moment we must be content with pointing out that should it ever prove possible to use wave-lengths of the order of 100 Å.U. we should have an instrument with a resolving power approaching that of the present electron microscope!

Very little is known concerning the absorption of cellular constituents in the far ultraviolet. It is true that most substances absorb very strongly below about 2,300 Å.U. Nevertheless, 'windows', or regions of transparency, are known to exist for some substances below 2,000 Å.U. It is not impossible that biologically important regions of specific absorption may exist at lower wave-lengths.

Absorption Spectroscopy of Cells

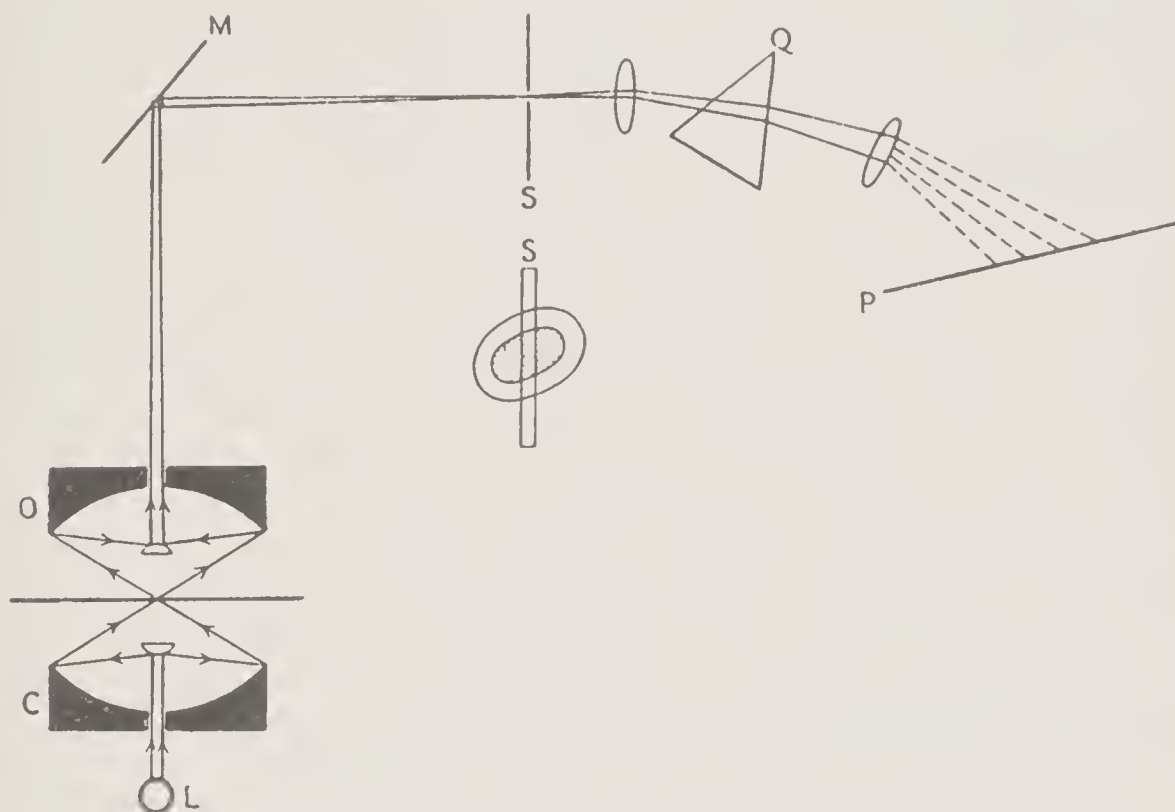
Many organic substances possess characteristic absorption bands in various parts of the spectrum which may aid in their identification. Microscopes with spectroscopic eyepieces have frequently been used for the study of pigments such as haemoglobin and its derivations, which have absorption bands in the visible region. Most unpigmented substances of biological importance absorb most strongly in the ultraviolet and infra-red regions. In some cases it is possible to locate and identify a substance by its ultraviolet absorption spectrum. The brilliant work of Caspersson^{32,33,34} laid the foundations for the applications of this method to microscopical objects such as living or fixed cells (see also Cole and Brackett³⁹; Hydén⁶³; Thorell¹¹²; Pollister and Ris⁹¹). In principle the method consists in taking photographs of the cell in a number of different wave-lengths in the ultraviolet. The photographic density of any given point in the cell is then measured, and a curve relating the absorption of light to the wave-length is drawn.

Valuable information concerning the distribution of nucleic acids (or strictly purine and pyrimidine compounds) and proteins has been obtained in this manner. Most of the difficulties in the technique arise from the fact that only quartz microscopes have hitherto been available for ultraviolet microscopy. As we have seen, quartz objectives are *monochromats* and are corrected for use at one wave-length only. In order to plot an absorption curve it is necessary to employ a relatively

wide range of wave-lengths (the important region for work on proteins and nucleic acids is 2,400–3,200 Å), so that some loss of accuracy is to be expected. Moreover, strictly monochromatic light (from a monochromator) must be used, and both the condenser and the objective should be refocused at each wave-length. In order to obtain sufficient points for a smooth curve, records in very many wave-lengths must be taken, and it is often necessary to use several different light sources (e.g. mercury arc, cadmium spark) in turn. Such a procedure is very laborious and may result in an unduly long exposure of the object to ultraviolet light, particularly if several trial focusing exposures have to be made. This is particularly undesirable in the case of living cells—indeed, Brumberg and Larionow²⁸ have claimed that the nuclear absorption of living cells differs from that of irradiated cells. It is not proposed to discuss the results and theoretical interpretations of the Caspersson school. Many controversial points have arisen as a result of this work and a proper verdict cannot be given at present. Nevertheless, whatever the future may reveal, one cannot do otherwise than express profound admiration of the tremendous theoretical skill and painstaking experimental work that have characterized Caspersson's writings.

There is no reason why a similar technique to that used by Caspersson should not be adopted in the case of the reflecting microscope. Monochromatic light of *any* wave-length, ultraviolet, visible, or infra-red, could be fed into the reflecting condenser and the absorption of the specimen at each wave-length measured. It is possible that this method will be used for some problems, but the method at present in use is rather simpler (Barer, Holliday, and Jope¹³). Instead of monochromatic light, the *entire spectrum* emitted by the source is passed through the specimen. A magnified image of the latter is then projected on to the slit of a spectrograph (Text-fig. 10). The action of the spectrograph is to split up the illumination falling on the slit into its constituent wave-lengths. Thus, a photograph of the slit, together with whatever is imaged across it, is obtained *simultaneously in every wave-length emitted by the source*. Fig. 2, Plate 2, shows the result obtained by this method using a mercury arc as source, with the image of part of a salamander's red blood cell focused across the slit. A monochromatic picture of the slit and of the part of the cell image across it is obtained *simultaneously* in every line of the mercury spectrum. Fig. 3, Plate 2, may illustrate this point rather better. It was obtained by projecting the images of several human red cells on to an unusually wide slit—so wide in fact that some of the images overlapped. It is proposed to call this type of record a 'spectromicrograph'. The spectromicrograph will often enable the main absorption bands of the specimen to be determined at a glance, but for quantitative work the variations of photographic density can be

measured by scanning the plate in a recording microphotometer. In the case of a line spectrum, a scan is carried out vertically across the cell at each wave-length. Text-fig. 11 illustrates the results of such a plot carried out at the different wave-lengths on a single frog's red blood cell. It will be seen that at $436\text{ m}\mu$ and particularly at $405\text{ m}\mu$ (near the Soret band of oxy-haemoglobin at $415\text{ m}\mu$) there is intense absorption in the

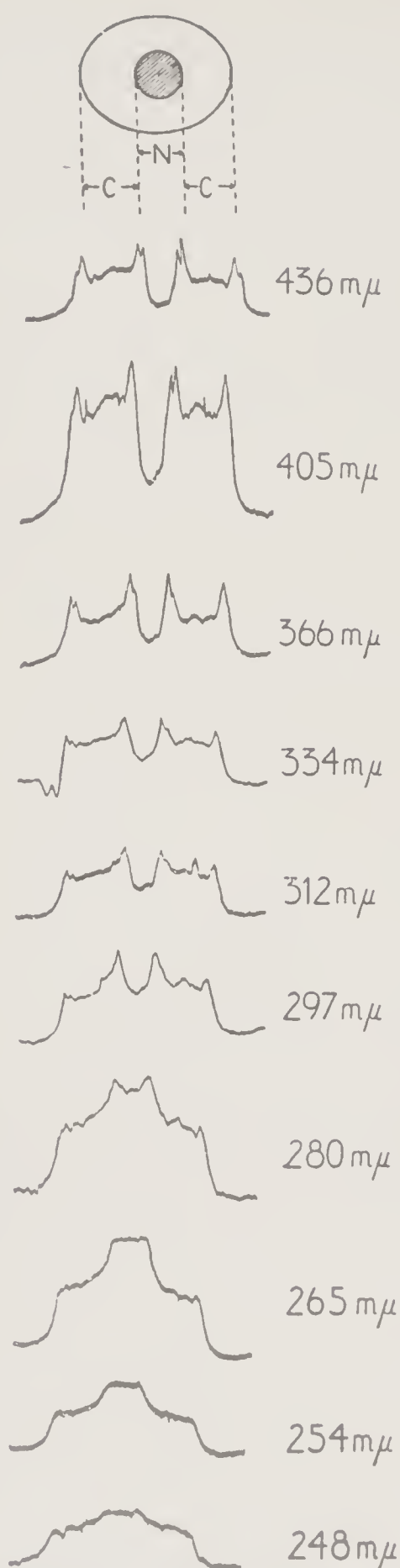


TEXT-FIG. 10. Ultraviolet spectromicrography with the reflecting microscope.

L. Light source. C. Condenser. O. Objective. M. Front-surface aluminized mirror. S. Slit of spectrograph. Q. Quartz prism. P. Photographic plate. An image of a projected cell is across the slit of the spectrograph.

cytoplasm whereas the nucleus shows almost no absorption. At $366\text{ m}\mu$, $334\text{ m}\mu$, and $312\text{ m}\mu$ neither cytoplasm nor nucleus show a special absorption. The nuclear absorption gradually rises below $297\text{ m}\mu$ until at $265\text{ m}\mu$ there is intense nuclear absorption but relatively low cytoplasmic absorption.

It will be appreciated that the ability to obtain a record in so many wave-lengths by a single exposure results in a great saving of time and labour. The ultimate triumph of achromatism is seen when a *continuous* source of radiation is used. Fig. 4, Plate 2, shows the spectromicrograph of a red cell using a hydrogen discharge tube. The latter emits practically continuous radiation between $2,000$ and $3,500\text{ \AA}$. It gives us virtually an *infinite* number of monochromatic pictures. A recording microphotometer plot can now be carried out in a longitudinal or a vertical direction. The longitudinal plot gives the variation with wave-length of the absorption of *any given point* in the cell, whereas the vertical plot gives the variation of absorption of *different* points in the cell at the *same*



TEXT-FIG. 11. Absorption of light by a frog's red blood cell at ten different wave-lengths (see text).

wave-length. The use of a continuous spectrum gives the completest possible picture of the light absorption characteristics of the cell. Such a method would be quite impracticable without a completely achromatic microscope system, for it is essential that all wave-lengths constituting the image should be in identical focus on the spectrograph slit. A source giving a continuous spectrum from the ultra-violet to the infra-red would be most valuable, but unfortunately none such exists. The tungsten lamp continuum extending upwards from the near ultra-violet is useful for some purposes, e.g. for demonstrating the Soret band in haemoglobin (Fig. 5, Plate 2).

An important advantage of the use of a continuous spectrum is that it enables us to observe *accurately* the absorption peaks of substances under the conditions within the cell, and to compare them with the same substances in free solution or in the solid state. Thus, the absorption peak of an amino acid may show slight shifts according as to whether it is in the form of the free acid, built into the structure of a protein, or within a cell. This type of information on the 'fine structure' of the absorption curve cannot be obtained by the use of line spectra alone, and provides essential basic data for quantitative cytochemistry.

Spectromicrography is an extremely sensitive method for demonstrating local concentrations of minute quantities of certain substances. To quote a specific instance the total amount of haemoglobin in a human red cell is

only 30×10^{-12} gm., yet it is possible to plot variations in its distribution within the cell. The method should be of use in the identification

of very small amounts of materials such as antibiotics, hormones, &c., during the early stages of chemical isolation. A tiny fragment less than 10μ in diameter and weighing less than 10^{-12} gm. is usually adequate for a spectromicrograph (Fig. 6, Plate 2). Numerous possibilities exist in the direction of new methods of biological 'labelling'. Thus it may prove possible to introduce substances having very characteristic absorption bands into living cells, to locate them within the cell and to follow their metabolic changes. Some of the carcinogenic hydrocarbons having absorption bands in the near ultraviolet which could not be confused with any bands due to substances normally present in the cell, are being investigated at present (Barer, Berenblum, and Jope¹²). Much of what we have said concerning the ultraviolet region applies in principle to the visible and infra-red regions also, but direct photographic methods of recording are not so easily available. Photographic densitometry in the visible region is complicated by the use of sensitizing dyes in the photographic plates. Standard photographic methods in the infra-red are only available as far as 13,000 Å, though indirect methods have been described which can be used for much longer wavelengths. In any case, there is no reason why the usual photoelectric or thermal methods should not be used. Infra-red spectroscopy has been of enormous service to organic chemistry—the infra-red absorption spectrum has been described as the 'finger-print of the molecule' and its extension to microscopical biological material might prove to be of considerable importance.

Working Distance

An important feature of the Burch reflecting microscope is its long working distance. When in focus, the distance between the mounting of the small mirror and the specimen is about 13 mm., and it could be increased to 20 mm. without much difficulty. This is very considerable as compared with the usual objective lens of equivalent NA, which has a working distance of the order of 0.5 mm. Even with an immersion component the working distance may be as great as 6 mm. This feature is of particular value in certain types of work such as micro-dissection. Not only does it enable the usual moist chamber and hanging drop preparations to be dispensed with, but it has enabled micro-dissections to be carried out on the surfaces of organs such as the spleen, kidney, brain, &c., exposed in the living anaesthetized animal. The writer has carried out such manipulations using various types of oblique illumination and quartz-rod illumination. There is no doubt that the scope of micro-dissection has been greatly extended by the reflecting microscope, and the development of auxiliary methods of illumination specially adapted to this purpose is highly desirable. Similar remarks apply to the use of the instrument as a high-power capillary and skin microscope.

Numerous other applications of the long working distance suggest themselves, such as observations on the effect of radiations, ultrasonic waves, and electric fields on living cells, and in fact almost any work in which it is desired to study cells or tissues in thick chambers or which necessitates the use of much apparatus concentrated in a small space around the object.

Future Developments

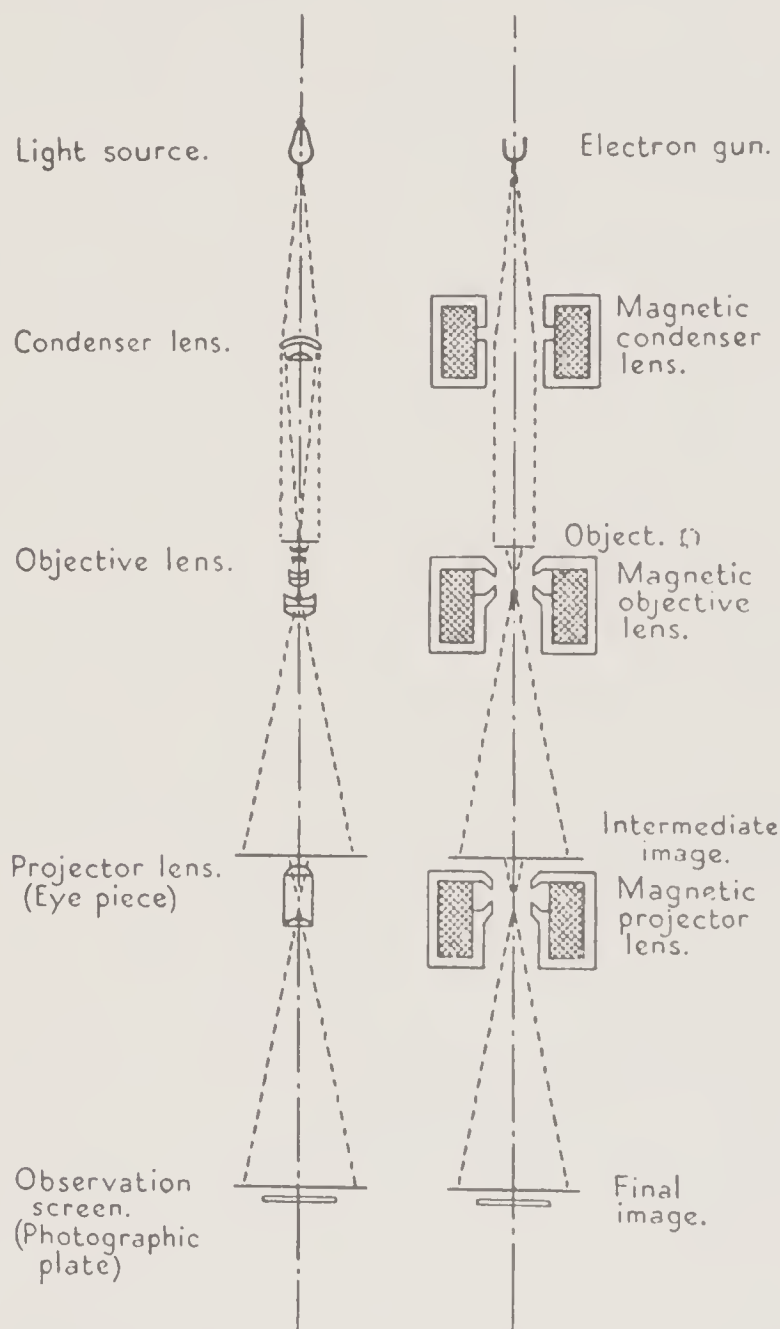
The writer has frequently been asked whether he believes that the reflecting microscope will ultimately replace the refracting microscope. There is no simple answer to this question. Much depends on the possibility of employing mass-production methods. At present, the construction of a high-performance reflecting microscope is very difficult and takes many months. Perhaps one of the most hopeful possibilities is that suitable mirrors might be made by moulding from a master die. Assuming that the mirrors could be made cheaply and of durable material, the reflecting microscope might very well compete with the lens microscope. As a result of over a year's experience with the Burch microscope, the writer is convinced that it is as adaptable and easy to use as any other type. The lens microscope will probably continue to hold its own as a routine instrument, but for research purposes we may look forward to the reflecting microscope of the future—an instrument of NA 0.98, increased to NA 1.4 by fluorite immersion components, adapted for use at any wave-length between 1,000 Å (or less) and 50 μ , with alternative phase-contrast, polarized light, or 'normal' illumination at all wave-lengths, and with facilities for absorption spectroscopy in any spectral range.

g. The Electron Microscope

As we have seen, the resolving power of a microscope is inversely proportional to the wave-length of the radiation employed. The possibility of using very short wave-length ultraviolet light has been referred to in our discussion of the reflecting microscope. Very much shorter wave-lengths can be attained by the use of charged particles such as electrons. According to modern physical concepts charged particles in motion can be regarded as associated with trains of waves. The wave-length of an electron is given by $\lambda = \sqrt{(150/V)}$ Å.U., where V is the accelerating voltage. Taking $V = 50,000$ volts, $\lambda = 0.05$ Å.U., i.e. about 100,000 times smaller than the wave-length of visible light. It is, indeed, fortunate that simple means of focusing electron beams exist, thus rendering possible the construction of an electron microscope. Electron beams can be focused by electrostatic or electromagnetic fields. Although electron microscopes have been constructed using both electro-

static and electromagnetic 'lenses', the latter are generally superior and are used in most commercial models.

A comparison of the lens systems of the optical microscope and the electron microscope is shown in Text-fig. 12. The superficial similarity



TEXT-FIG. 12. Comparison of optical and electron microscopes.
(Courtesy of Metropolitan-Vickers Ltd.)

is very striking. The final image in the electron microscope is recorded photographically or can be observed visually on a fluorescent screen. Whereas in the conventional optical microscope contrast is obtained by variations of intensity due to differential absorption of light by different parts of the specimen, in the electron microscope contrast is produced by electron *scattering*, rather than absorption. All matter scatters electrons, in proportion to its mass. Electrons are scattered in passing through an object, and those electrons which are scattered at a

sufficiently great angle will not enter the aperture of the objective and will play no part in the formation of the image.

If it were possible to employ electron lenses as well-corrected as are optical lenses the theoretical resolution of the electron microscope would be of the order of $0.03 \text{ \AA.U.}$ Unfortunately, technical difficulties make it impossible to design electron lenses even moderately free from aberrations. Spherical aberration is particularly serious in electron lenses and necessitates the use of very small apertures. Chromatic aberration arises from the fact that electrons of different velocities are brought to different foci. This factor can be largely controlled by ensuring a very stable current and voltage supply. For a full discussion of aberrations see Zworykin *et al.*¹¹⁸ The result of these aberrations is that the best resolution of present instruments is of the order of $20\text{--}30 \text{ \AA.}$ Such resolutions are only obtainable with suitable high-contrast specimens and under optimum conditions, and, in practice, with biological material the maximum resolution usually lies between 50 and $100 \text{ \AA.}$ Resolution as good as $8.5 \text{ \AA.}$ has been reported by Hillier⁶¹ using a special lens and electron gun. Although there does not seem to be any immediate prospect of any great improvement in resolving power of the electron microscope, it must be admitted that a resolution of $100 \text{ \AA.}$ and a useful magnification of up to $100,000$ times makes it a very powerful research tool. Several standardized commercial models are available. Examples of these are the Metropolitan-Vickers EM2 and EM3 models, made in this country, and several models produced by RCA in America. An important new model has been introduced by Phillips. The Metro-Vick EM3 model uses a 100 K.V. power supply, and the magnification can be varied continuously from $1,000$ to $100,000$ times. Stereoscopic photographs can be obtained by a mechanism which tilts the specimen holder, and the instrument is readily adaptable for electron diffraction work. In many respects the operation of such instruments is no more difficult than that of an optical microscope. Skilled maintenance is, however, essential. One of the most serious disadvantages of the electron microscope, from the biological point of view, is that, in general, the specimen has to be placed in a vacuum. This itself must be expected to produce gross changes in the structure of protoplasm. In addition the action of the intense electron beam may produce distortion due to heating, charging, and actual tearing of the specimen. Extreme caution is, therefore, necessary in interpreting the appearances of biological objects under the electron microscope. The use of 'electron stains'—substances such as osmium tetroxide or phosphotungstic acid which can be used to increase contrast in some specimens—is also open to similar objections. There can be little doubt that much misleading work has been published, and a more critical attitude must be adopted if the method is not to fall into disrepute. Despite these criticisms much valuable information has been

obtained. The work of Hall, Jakus, and Schmitt⁵⁷ on the structure of striated muscle fibres has been extremely useful in confirming the presence of several striations and enabling the presence and orientation of myosin fibrils to be observed. The same workers (Schmitt, Hall, and Jakus¹⁰⁴) have also shown that collagen fibrils show regular close striations at intervals of 640 Å.

An interesting study of the morphology of chick tissue culture cells has been carried out by Porter, Claude, and Fullam⁹². Observations were made on the cytoplasm and mitochondria and the effects of various fixatives were observed. The possibility of artefacts was constantly borne in mind. Isolated mitochondria have also been studied by Claude and Fullam³⁸. In the bacteriological field, the electron microscope has revealed details of the morphology of numerous viruses, bacteriophages, and bacteria (Mudd and Anderson⁸¹; Green, Anderson, and Smadel⁵⁰; Luria, Delbruck, and Anderson⁷³; Dawson and McFarlane⁴²). Several biochemical investigations of interest to cytologists have been carried out. These include the study of actin and myosin (Astbury, Perry, Reed, and Spark⁵; Jakus and Hall⁶⁴), and of fibrin (Wolpers and Ruska¹¹⁴). Space does not permit a fuller account of the numerous applications of the electron microscope. The reader is referred to the bibliography for more details.

Future Developments. We have already seen that the resolution of present instruments is very much below the theoretical maximum. There is no doubt that considerable improvement is possible but an entirely new approach will have to be made before apertures of the order of those employed in the optical microscope can be attained. Another possibility is the use of particles of shorter wave-length than the electron, e.g. atomic ions. The proton or hydrogen ion is about 1,840 times as heavy as the electron, and has a wave-length 43 times shorter. An electrostatic proton microscope is now being built at the Collège de France. Its value in biological research may be limited as the penetrating power of the proton is very low.

Quite apart from the question of increasing resolving power, improved methods of preparation of specimens are required. At the moment the maximum permissible thickness of the specimen is limited to about 1,000 Å (0.1μ). Contrast tends to be lost above this. Relatively few biological specimens are suitable for direct electron microscopy. Some progress is being made in the development of high-speed microtomes capable of cutting sections less than 0.1μ in thickness (Richards *et al.*⁹⁶; O'Brien and McKinley⁸²), but some degree of distortion appears to be inevitable. One solution may be the use of much higher accelerating voltages, with greater penetrating power, enabling the use of thicker specimens. Another promising technique is the examination of a durable replica of the surface of the object. Various substances have been

employed, such as Formvar, collodion, and methyl methacrylate-silica (Brown and Jones²⁶). The appearances of such replicas are not always easy to interpret (see Barnes, Burton, and Scott¹⁹, for a very good example of optical illusion in replica photography). Another useful method is that of 'shadow-casting' whereby the specimen is coated obliquely by a thin layer of metal such as gold or chromium (Williams and Wyckoff¹¹³). Such indirect methods are, however, time-consuming and tell us little of the internal structure of the specimen. (Reed and Rudall⁹⁴ have recently described a promising 'layer-stripping' technique.) There appears to be little possibility of examining *living* material by present methods. Von Ardenne and Friedrich-Frekse⁴ claimed to have observed the germination of spores of *Bacillus Vulgatus* after examination in a 180 K.V. electron microscope, but this is an exceptional instance. A thin-walled specimen chamber enabling the use of water has been described by Abrams and McBain¹, but as a film of water 1μ thick is opaque to electrons it has very limited application. Another possibility is the use of 'shadow' microscopes in which the specimen is made to cast a shadow in a beam of X-rays originating from a print source formed by focusing a beam of electrons on a fine metal foil. The specimen need not be dehydrated or in a vacuum, but the practical application is limited by the low intensity of the X-ray beam. If the writer has been more concerned with a discussion of the limitations rather than the achievements of the electron microscope, it is because he feels that these points are not as fully appreciated as they should be. Increased magnification and an entirely new type of contrast have brought their own peculiar difficulties in interpretation, quite apart from the possibility of artefacts. At the same time when we remember that the electron microscope is at the same stage of development as the optical microscope before the invention of the achromatic lens there are real grounds for optimism.

h. Conclusion. The Future of Microscopy

Only twenty years ago it seemed as if the microscope had reached almost its final stage of development. To-day the position is very different. Powerful new methods are being developed in every part of the world, and it seems as if we are witnessing the dawn of an inspiring Golden Age of Microscopy. It appears that most progress will be made by using the microscope as a kind of interferometer rather than as a mere magnifying glass. It is a great tribute to Abbe that for all its shortcomings, his theory of image formation should provide the basis for the development of new techniques—in electron optics as well as light microscopy. On the purely technical side we may look forward to the use of new glasses and plastics, a more universal use of aspherical surfaces, anti-reflection coating of lenses, and other processes which will

undoubtedly lead to considerable improvements in performance. The microscopist of to-day is indeed fortunate, for to him especially apply the words of Henry Baker, F.R.S., written 200 years ago in the introduction to his book, *The Microscope Made Easy*:

That Man is certainly the happiest, who is able to find out the greatest Number of reasonable and useful Amusements, easily attainable and within his Power: and, if so, he that is delighted with the Works of Nature, and makes them his Study, must undoubtedly be happy; since every Animal, Flower, Fruit, or Insect, nay, almost every Particle of Matter, affords him an Entertainment. Such A Man never can feel his Time hang heavy on his hands, or be weary of himself, for want of knowing how to employ his Thoughts: each Garden or Field is to him a Cabinet of Curiosities, every one of which he longs to examine fully; and he considers the whole Universe as a Magazine of Wonders, which infinite Ages are scarce sufficient to contemplate and admire enough.

Addendum

Progress in Microscopy has been extremely rapid and some portions of the above article require amendment. Of recent advances pride of place must be given to Gabor's remarkable theory of 'Wave-front reconstruction microscopy'. (D. Gabor, *Nature*, **161**, 777, 1948. A full account will appear in *Proc. roy. Soc. A.*) The importance of this work lies not so much in its possible practical applications as in its brilliant conception of the mode of image formation. It is undoubtedly the most important advance in wave theory since Zernike's development of phase-contrast microscopy. The original papers must be consulted for an account of the theory, but the chief practical application, on which experimental work is now in progress, is in electron microscopy. It suggests a method whereby the resolution of the electron microscope may be improved to something of the order of 1–2 Å.U. without having to reduce the aberrations of electron lenses. Even if these experiments fail, Gabor's theory is nevertheless assured of a lasting place in Microscopy.

The phase-contrast microscope has now become almost a routine instrument in many fields of work, and several firms are now producing new models. A special instrument allowing full control of both phase and amplitude (Cooke, Troughton, and Simms) has been tested in the writer's laboratory. Broadly speaking, it was found that the ability to vary the phase was not of very great value, as retardations of $\frac{1}{4}\lambda$ or thereabouts were most universally useful, but amplitude variation over a fairly wide range and the ability to change rapidly from positive to negative phase contrast were helpful in many problems. A simple variable amplitude instrument should fulfil many needs.

Work on the applications of the Burch reflecting microscope has also

continued. The extension of spectromicrographic methods to the infra-red region, foreshadowed in the above article, has now been carried out (Barer, Cole, and Thompson, *Nature*, **163**, 198, 1949; Barer, *Lancet*, 1949, p. 533). The Burch microscope has now been used over a spectral range extending from 2,200 Å.U. in the ultraviolet to 14μ in the infra-red. A survey of the possible applications of infra-red spectromicrography to biological material is now in progress. A study of dichroism of crystals and fibres has also been carried out using polarized light both in the ultraviolet and infra-red. A full review of work on the reflecting microscope will be given in a forthcoming issue of the *Journal of the Royal Microscopical Society*.

Another type of infra-red microscopy recently investigated by the writer has involved the use of photo-electric image converters working at 18,000 volts. This has rendered possible the actual visualization of infra-red images at a wave-length of about 1μ . The enormous advantage of being able to see the infra-red image without all the attendant difficulties of focusing and photography will be appreciated. Infra-red phase-contrast microscopy has been carried out in this manner with interesting results. Image converters may have many uses in microscopy, since it is possible to increase the brightness of very faint images. An image converter for use in ultraviolet microscopy would be extremely valuable.

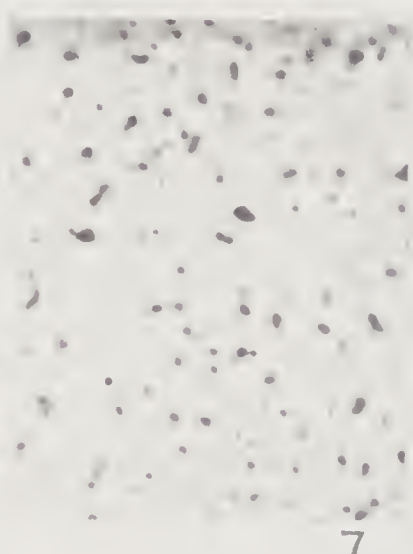
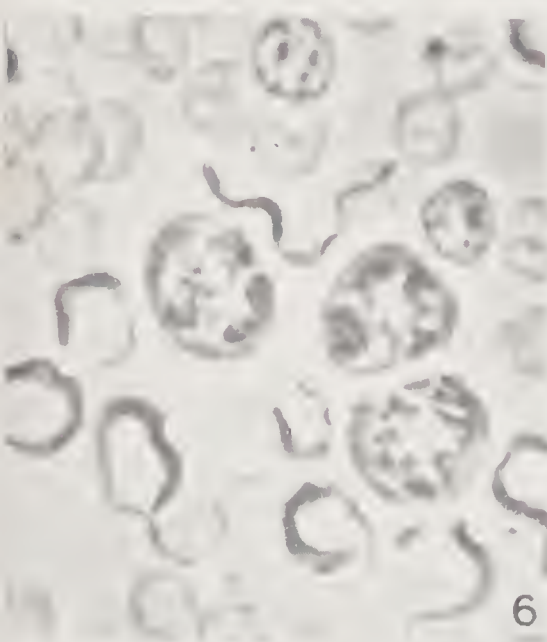
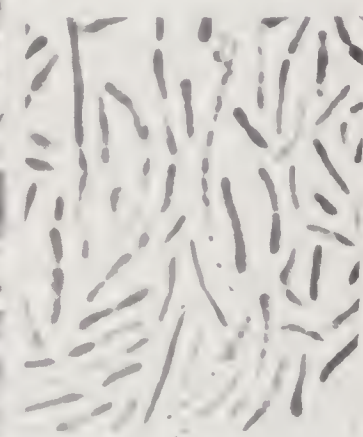
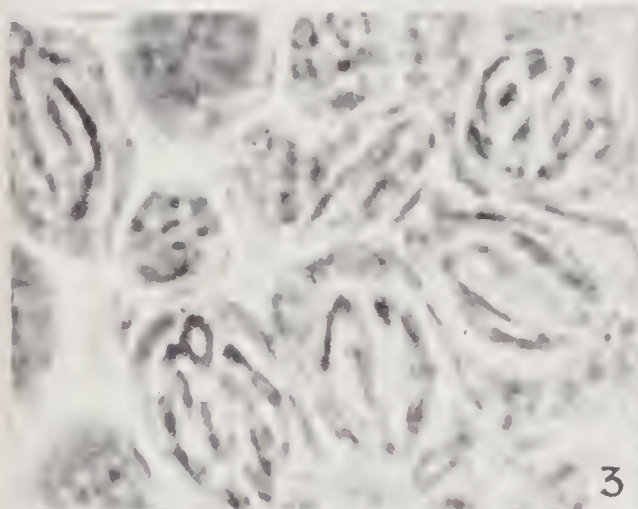
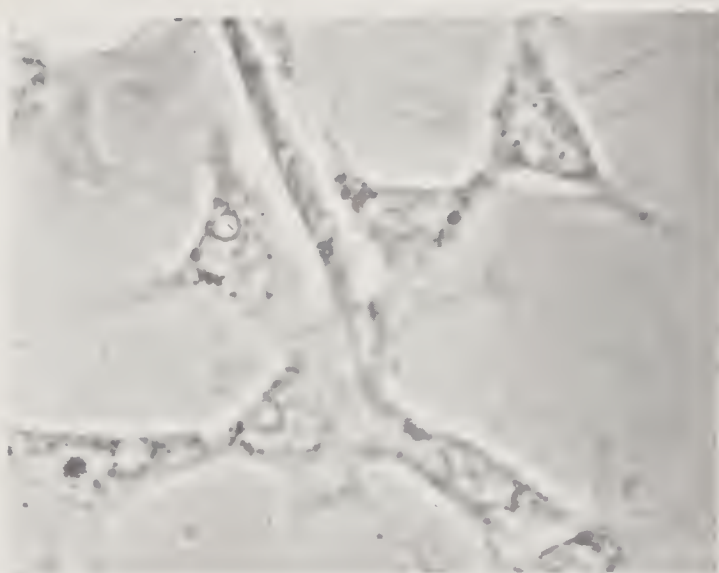
An ingenious scheme for increasing the working distance of an objective has been proposed by J. Dyson (*Nature*, **163**, 400, 1949. A full account will appear in *Proc. Phys. Soc.*) This is essentially a reflecting microscope of unit magnification, the image from which can be examined by an ordinary objective. The writer has had the opportunity of testing this apparatus, and there is no doubt of its efficacy. Working distances of up to 34 mm. can be achieved with an NA of 0.5. A rough trial showed that the system functioned satisfactorily with phase-contrast illumination.

An illustration of the way in which new advances can be made even in fields which have apparently been fully explored is the construction by Boutry and Billard (*C.R. Acad. Sci. Paris*, **224**, 1496, 1947) of a new type of 'polychromatic' lens objective. Instead of attempting to achieve achromatism, spherical aberration and coma have been corrected for one wave-length, and by suitable design have been kept fairly constant over a wide range of wave-lengths. The objectives are made of one material only and in the simplest form consist of uncemented doublets. It is essential to use monochromatic light, but unlike the usual monochromatic objectives there is no deterioration in the image over a wide spectral range. The objectives have an exceptionally large angular field, which makes the use of an ordinary eyepiece impossible. Nevertheless, this feature should be useful in photomicrography. Objectives of this type may have great possibilities for ultraviolet work.

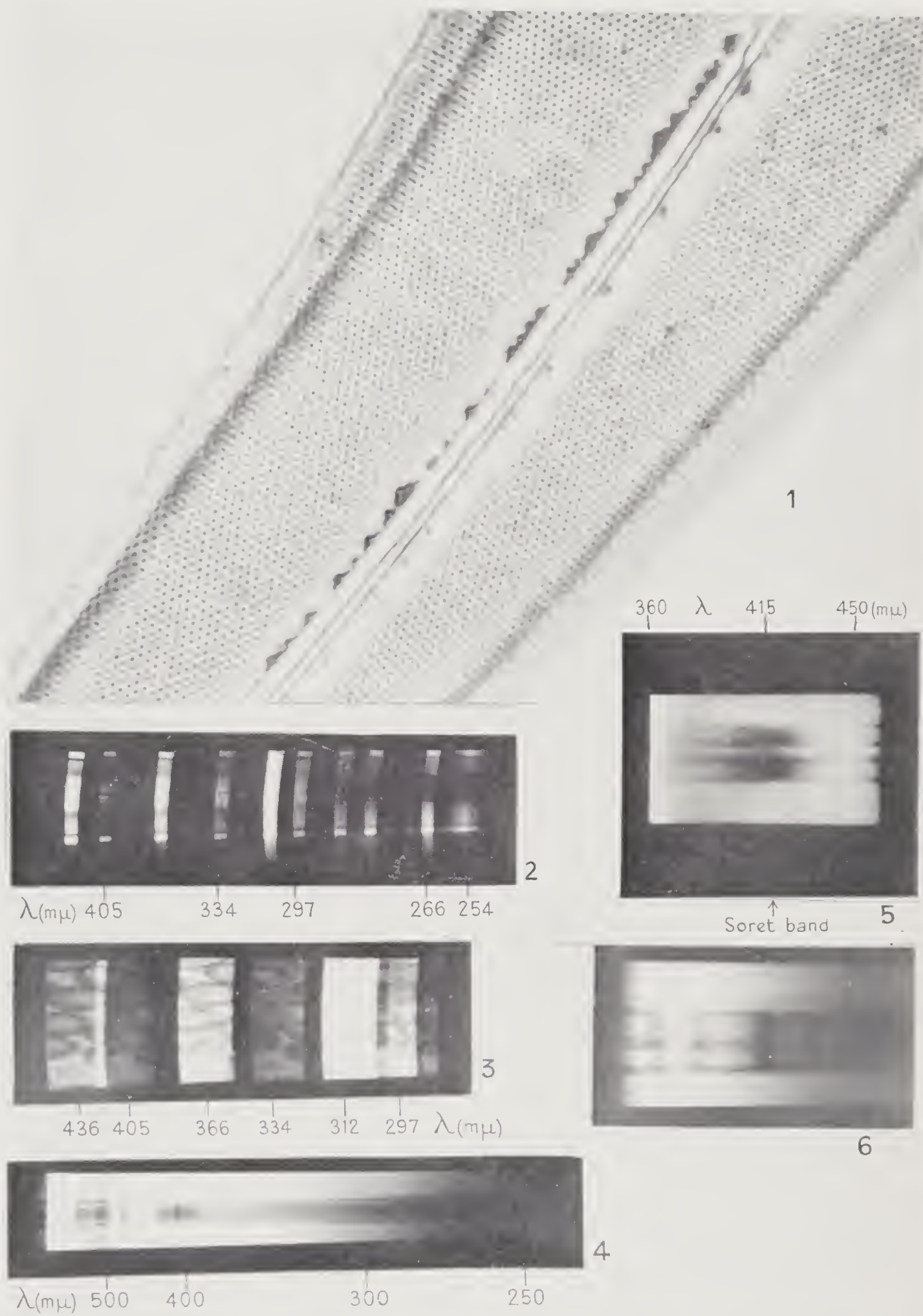
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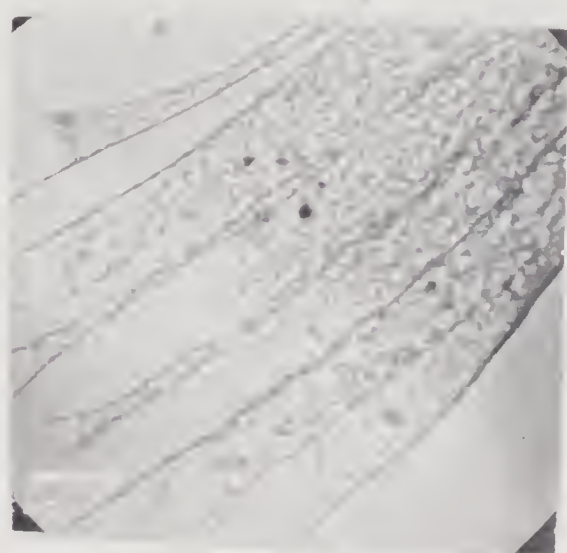
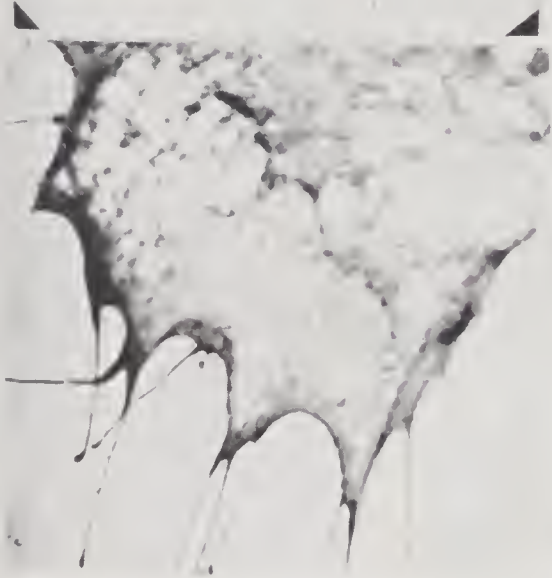
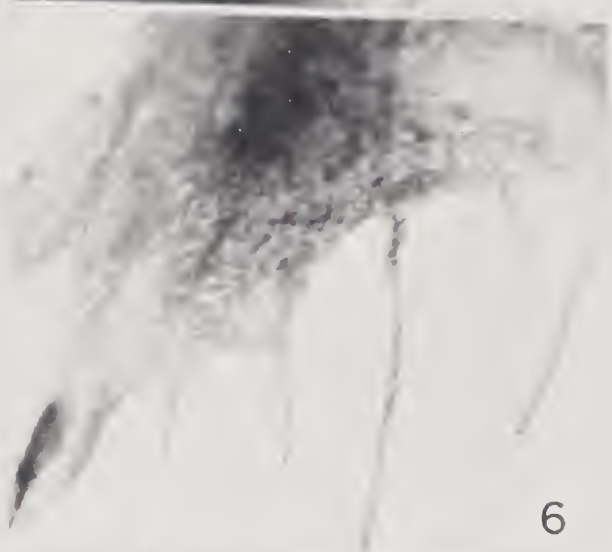
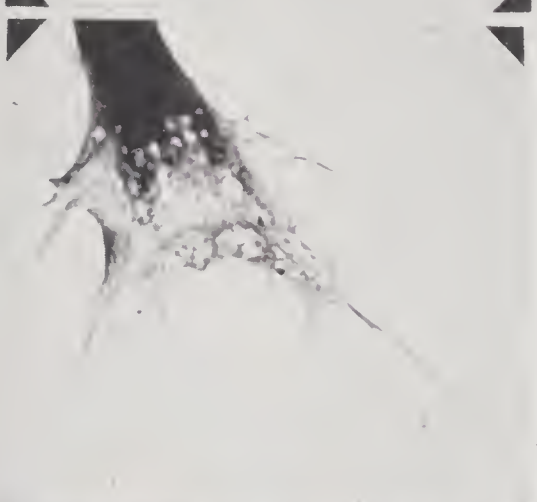
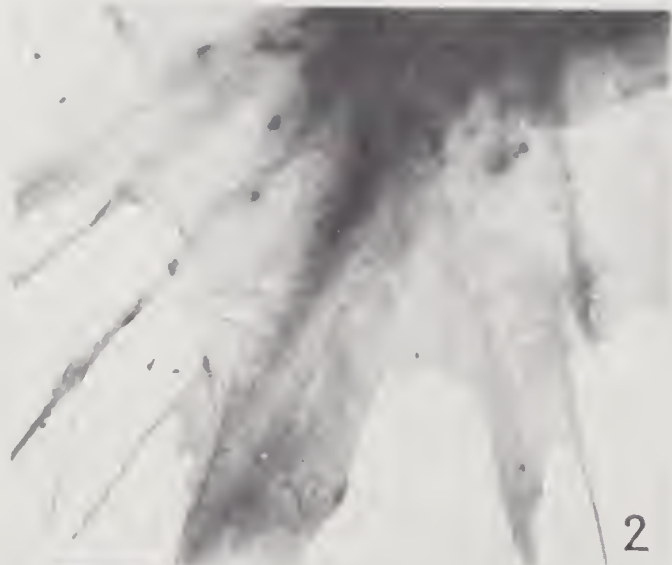
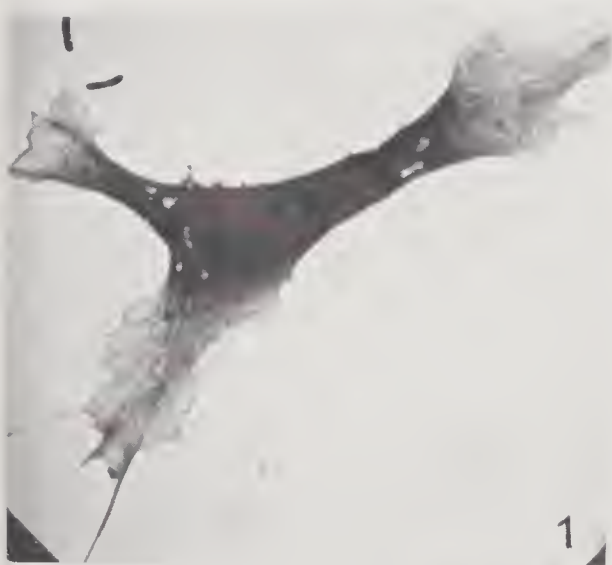
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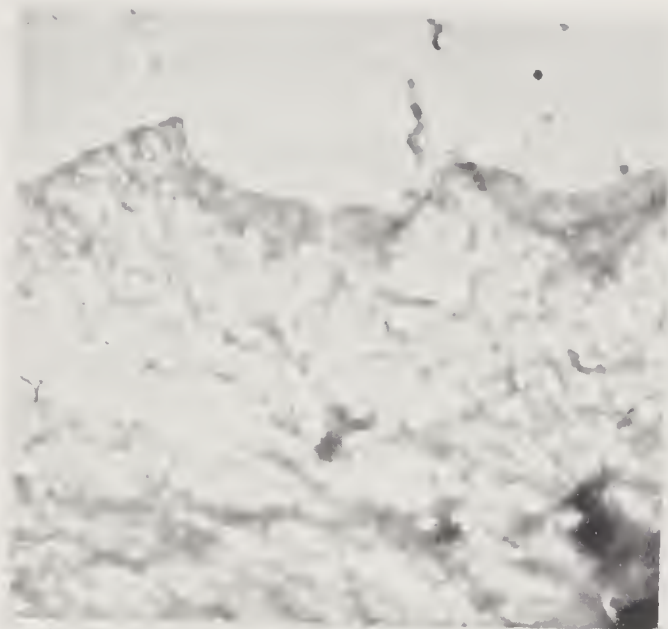
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DESCRIPTION OF PLATES

PLATE 1

FIG. 1. Part of cell of salivary gland of *Chironomus*, showing nucleus, nucleolus, and chromosomes. Fresh, unstained. Phase-contrast NA 0.65×600 .

FIG. 2. Living cells in tissue culture (snail amoebocytes). Note intracellular granules and delicate protoplasmic processes. Phase-contrast NA 0.65×600 .

FIG. 3. Living cells from testes of salamander, showing chromosome patterns. Phase-contrast NA 0.65×600 .

FIG. 4. Red blood corpuscles of salamander. Note the remarkable banded appearance, which has also been observed in other amphibian red cells. Phase-contrast NA 0.65×600 .

FIG. 5. Living bacteria (*B. megatherium*). Note the intracellular bodies, which are a prominent feature in some species. Phase-contrast NA $1.30 \times 2,000$.

FIG. 6. Trypanosomes in a blood smear (unstained). Phase-contrast NA $0.65 \times 1,000$.

FIG. 7. Unstained preparation of Psittacosis virus particles. The larger black masses are probably clumps of virus, the pale grey bodies are the individual elementary bodies. Phase-contrast NA $1.30 \times 1,600$.

FIG. 8. Living bacteria (*B. Lactis aerogenes*) growing in the presence of streptomycin. Note the bizarre forms with intracellular bodies. Phase-contrast NA $1.30 \times 1,500$.

PLATE 2

FIG. 1. Diatom, *Pleurosigma Angulatum*, NA $0.65 \times 2,000$. This figure, and Fig. 7, Plate 1, illustrate the resolution obtainable by the phase-contrast method. The resolution is probably no better than that obtainable by conventional methods, but the improved contrast renders the detail more easily visible.

FIGS. 2-6. Spectromicrography with the reflecting microscope.

FIG. 2. Spectromicrograph of nucleated red cell (salamander). Mercury arc. The nuclear region is in the centre of each line. Note that the nucleus appears white (low absorption) at wave-lengths above $297\text{ m}\mu$, and dark below this. Note also the intense cytoplasmic absorption (due to haemoglobin) at $405\text{ m}\mu$.

FIG. 3. Spectromicrograph of a group of human red cells, taken with a very wide spectrograph slit. Mercury arc. This illustrates the fact that the record obtained is a set of simultaneous monochromatic photomicrographs. It will be observed that owing to the variations in intensity in the spectral lines, some wave-lengths appear to be over-exposed, others under-exposed. It is usual to take two or three exposures in order to allow for this.

FIG. 4. Spectromicrograph of nucleated red cell (frog). Hydrogen discharge lamp. This illustrates the type of record obtained with a continuous spectrum. It enables the *exact* wave-lengths of absorption bands to be determined.

FIG. 5. Spectromicrograph of nucleated red cell (frog). Tungsten filament lamp. This lamp gives a useful continuum in the infra-red, visible, and near ultraviolet regions. It is valuable for work on blood cells as it covers the range of the important Soret absorption of haemoglobin. This is seen in the figure as a dark area in the cytoplasm.

FIG. 6. Spectromicrograph of a microscopic crystal (weight approx. 10^{-12} gm.) of 1, 2, 5, 6, dibenzanthracene. Tungsten lamp. Wave-length range approximately that shown in Fig. 5.

PLATE 3

PLATES 3 and 4 show electron microscope photographs of snail amoebocytes grown in tissue culture (Barer, Crawford, and Pease, 1949). The objects of this work are (a) to study the morphology of invertebrate cells with the electron microscope, (b) to study the action of fixatives and other physical and chemical reagents on cell ultra-structure. These photographs illustrate the type of information obtained and some of the problems encountered with this technique.

FIG. 1. General low-power view of formalin fixed cell $\times 1,400$. The peripheral cytoplasm and delicate fibrils are well seen but the nuclear area is too dense to allow any detail to be seen. Note the small white 'holes' in the perinuclear region.

FIG. 2. View of peripheral cytoplasm in cell fixed with osmium tetroxide vapour $\times 4,000$. Note finely granular appearance of the cytoplasm, characteristic of osmic fixation. The cell margin, though sharp in outline, shows no features which suggest the existence of a morphological cell membrane. A few dense black bodies are seen in the cytoplasm, particularly in the perinuclear region.

FIG. 3. Nuclear region of osmium fixed cell $\times 2,700$. In this case some indication of nuclear structure was obtained but owing to the thickness of the nucleus the detail is hazy. It may be possible to obtain better nuclear detail by the use of very high voltage electron microscopes (see Dorsten, Oosterkamp, and Le Poole, *Phillips Technical Review*, 9, 193, 1947).

FIG. 4. This shows the dense black perinuclear bodies characteristic of osmic fixation.

FIG. 5. View of peripheral region of a cell fixed in formalin and treated with a weak solution of Lanthanum nitrate. $\times 1,800$. Note the delicate protoplasmic fibrils. Compounds containing heavy metals, such as phosphotungstic acid, phosphomolybdic acid, &c., are often used as 'electron stains' for increasing contrast in biological materials.

FIG. 6. Cytoplasm of cell fixed in formalin and then treated with osmium tetroxide vapour. Note the finely granular appearance. The cytoplasmic processes in this case are rather broad and appear to have almost a striated structure. $\times 4,000$.

FIG. 7. Periphery of cell fixed in formalin and 'stained' with Lanthanum nitrate. This illustrates a fairly common appearance, namely a rather dense region of cytoplasm at the cell margin (see also Plate 4, Figs. 1 and 8). $\times 4,400$.

FIG. 8. Cytoplasm of cell fixed in picric acid, $\times 7,000$. Note the network appearance and the absence of a definite cell edge. This method of fixation undoubtedly produces gross precipitation artefacts.

PLATE 4

FIG. 1. View of cytoplasm near edge of cell after fixation in 1 per cent. chromic acid. Note interlacing network appearance and denser zone at periphery. $\times 7,000$.

FIG. 2. High-power view of perinuclear 'holes' in cell fixed in formalin vapour. $\times 21,000$.

FIG. 3. High-power view of dark perinuclear bodies in cell fixed in osmium tetroxide. Some of these bodies appear to be joined together by long narrow strands. $\times 16,000$.

FIG. 4. The difference in appearance of the perinuclear region in osmium and formalin fixed cells is very striking and difficult to interpret. In general, osmium fixation produces the appearance of dark perinuclear bodies, whereas formalin fixation results in the formation of perinuclear 'holes'. The exact relationship of the dark bodies to the 'holes' is at present undecided. This figure shows an appearance occasionally seen in cells which have been fixed in formalin and then impregnated with osmium. It suggests a mixture of dark bodies and 'holes'. Note that one dark body appears to lie in the centre of a 'hole'. $\times 8,000$.

FIG. 5. Another cell in which a mixture of bodies and 'holes' is found. This cell was fixed in formalin vapour and is a rare appearance. $\times 11,000$.

FIG. 6. View of 'holes' in a formalin fixed cell shadowed with chromium. $\times 8,000$. This technique shows that the white areas seen in a transmission photograph are, in fact, crater-like spaces. The depth of the craters can be calculated from the length of the shadows, and is approximately 0.13μ . The entire question of the nature of the perinuclear inclusions in these cells is at present under investigation. Experiments carried out with phase-contrast microscopy suggest that the 'holes' may be drying artefacts. Nevertheless, the regularity with which these 'holes' appear would seem to indicate that they are formed in relation to some definite structure normally present within the cell. The problem affords a good example of the way in which artefacts may arise and of the need for caution in the interpretation of electron microscope images.

FIG. 7. Peripheral fibrils of cell fixed in formalin and shadowed with gold-manganin. The height of the fibrils is calculated to be about $250 \text{ \AA.U.}$ (0.025μ). Fibrils as thin as $150 \text{ \AA.U.}$ have also been observed. It must be remembered that considerable shrinkage may occur as a result of drying. $\times 14,000$.

FIG. 8. View of periphery of cell fixed in formalin and shadowed with gold-manganin. This technique enables us to shed light on the problem of the nature of the dense cell margin (see also Fig. 7, Plate 3). The distribution of the shadow makes it certain that this margin is, in fact, a raised rim in some cells. The direction of shadowing is from top left to bottom right. Note that in some places this has resulted in a shadow being cast on the cell itself. The height of the cell rim in this case is about $600 \text{ \AA.U.}$ (0.06μ). The presence of a raised rim does not exclude the possibility suggested by Porter *et al.*⁹² that there may be a dense ectoplasmic layer as well, but makes this suggestion less likely.

CHAPTER II

PHYSICAL AND PHYSIOCHEMICAL STUDIES OF CELLS

By J. F. DANIELLI

i. INTRODUCTION

IN this chapter typical examples are given of the results obtained by investigating cells by physical methods. Our endeavour is not to be exhaustive, but to show the types of questions which may be answered by physical methods and to indicate some of the limitations of these methods. In conclusion we shall discuss the use of models of biological systems, again not exhaustively, but mainly in the light of relatively new knowledge of colloids and surfaces. In doing so we do not wish to convey the view that a superficial similarity of behaviour of some biological systems and certain models affords any large measure of plausibility to the conclusion that the underlying mechanisms are the same in the two cases, but rather to show how the feasibility of hypotheses about biological systems may be tested, in the absence of sufficient detailed knowledge of the biological system for proof of existence to be obtained. For example, when we postulate that the plasma membrane of an *Arbacia* egg is a spherical shell of lipid material, we are assuming that such shells are stable. This assumption must be tested by the construction of artificial spherical shells of lipid. That artificial shells are stable shows us that the postulated structure is a *possible* one, though it tells us little about the probability that this structure actually exists in the egg.

ii. RADIATIONS

a. The Uses of Radiations

The earliest observations in the realm of cytology were made with visible light, and the development of the field has been intimately linked with progress in microscopical methods. In consequence the use and merits of the refracting microscope are too well known to need discussion here. Recently, however, as a result of the enterprise of Burch, a small number of reflecting microscopes have become available. Magnification is achieved in a reflecting microscope by the use of curved mirrors, which results in two important practical advantages over the refracting microscope: the working distance between the object and the mirror equivalent of the objective is of the order of a centimetre, even with a numerical aperture of 1.4 and optimal condenser conditions; and

when once in focus for light of one wave-length, the object is in focus for all wave-lengths from the far ultraviolet to the infra-red: there is no chromatic aberration. Comparatively little work has been done with this instrument to date, but much may be expected of it.

Of recent years much attention has been paid to the use of polarized light, interference (phase-contrast) methods, ultraviolet light, X-rays, and electron beams. The necessity of using radiations of different wave-lengths is thrust upon us because (1) a body absorbs light to an extent which varies with wave-length, (2) a body has different refractive indices at different wave-lengths, and (3) the limit of resolution is about half the wave-length of the form of radiation employed. Thus the theoretical limits of resolution are roughly: visible light 0.25μ , ultraviolet light 0.1μ , electron beams $0.01\text{ m}\mu$, X-rays $< 0.01\text{ m}\mu$.* Owing to practical limitations electron beams can at present be used down to about $4\text{ m}\mu$ only, but as X-rays are useful up to a limit of $50\text{ m}\mu$ or more, the whole range from macroscopic bodies down to molecular dimensions is now open for study.

The use of ultraviolet light extends the range of vision by comparatively little, but nevertheless has shown some protoplasmic structures not readily studied by ordinary light, such as the spiralization in prophase of somatic chromosomes in certain plants (Manton), and in the hands of Caspersson has been of much value for cytochemical studies, chiefly of the distribution of nucleic acid in cells. Caspersson pointed out that the pyrimidine rings of nucleic acid have an exceptionally high capacity for absorbing light in the ultraviolet at about $2,600\text{ \AA}$. Thus the detail seen in a photograph taken at $2,600\text{ \AA}$ is mainly determined by the distribution of purine and pyrimidine, which are believed to be mainly present in cells as nucleic acid. By careful investigation of the necessary instrumentation, Caspersson has found it possible to develop quantitative microspectrophotometric methods which, taken at their face value, permit one to calculate the exact amount of nucleic acid in a particular region of the cell with an overall error of the order of ± 10 per cent. There are, however, at least three remaining difficulties to be overcome before the quantitative data can be evaluated. First, the technique measures absorption by purine and pyrimidine, which may be present in other bodies in addition to nucleic acid. Thus J. Panijel has recently found purine forming an integral part of a protein isolated from *Ascaris* sperm, which thus has an absorption spectrum similar to that of a nucleoprotein, but which contains no nucleic acid. Consequently supplementary techniques are necessary for determining the type of molecule in which the purine and pyrimidine

* These magnitudes may be compared with the diameter of, for example, red cells 10μ , *B. coli* 2μ , vaccinia virus $150\text{ m}\mu$, tobacco mosaic virus $30\text{ m}\mu$, ovalbumin $5\text{ m}\mu$, cholesterol molecule $1.0\text{ m}\mu$, water molecule $0.3\text{ m}\mu$.

are incorporated, as was pointed out by Danielli earlier⁵⁴. Second, it is necessary to make a correction for light scattering in the specimen under examination. This is usually done by assuming that the scattering varies as λ^{-4} . But it is known that, in some systems at least, the variation is a function of particle size, varying as λ^{-2} for particles of the order of $10\text{ m}\mu$ or smaller, to λ^{-4} for particles of the order of $1\text{ }\mu$ or larger. This is a range of particular importance in cytology. Third, it is not known whether an area consisting of a mosaic of nucleic acid and other material, in which the nucleic acid is in particles which are below the resolving power of the wave-length used, will absorb as though the nucleic acid were uniformly distributed. Until these problems have been solved the main value of the ultraviolet microscope will lie in its use for microphotography. In this field many valuable results have been obtained, for example the distribution of nucleic acids in the chromosomes, nucleoli, and cytoplasm of cells, and changes in this distribution caused by physiological activity and disease. Much of this work has been summarized recently^{148, 149}.

At present, little has been attempted with infra-red microscopy. But it is a field which merits serious attention.

The purpose of using polarized light is rather different: it enables us, though still using visible light, to detect birefringent regions,* i.e. regions in which there are certain types of regular molecular or micellar patterns. Picken has given an excellent review of this field up to 1940¹⁵⁴. As examples of the use of polarized light we may take studies on tobacco mosaic virus, on chromosomes and cell division, and on muscle.

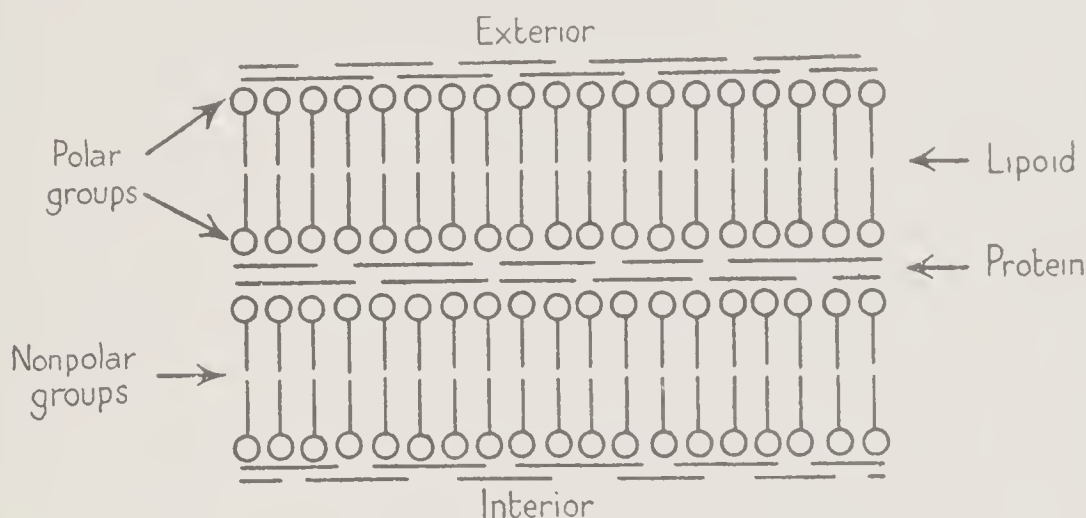
Concentrated suspensions of some colloidal particles are spontaneously birefringent. With tobacco mosaic virus, combined birefringence and X-ray studies show that in the spontaneously birefringent suspensions, which are formed under certain conditions, there are present needle-shaped virus particles which are oriented with their long axes parallel to one another (Bernal and Fankuchen¹³). The position of the needles is maintained by long-range forces whose nature is unknown.

The chromosomes of many species are birefringent, and Schmidt¹⁶⁰ showed that the chromatin micelles of the chromosomes of *Psamechinus miliaris* are negatively birefringent, but that the micelles are arranged in the chromosomes in such a way as to make the chromosome positively birefringent with respect to its long axis. The mitotic spindle and asters are birefringent, and the birefringence of the spindle is not lost on damaging the cell until the spindle swells. This suggests very strongly that the spindle is an aggregate of long micelles oriented parallel to its length—a view which is supported by the fact that on dehydration the spindle changes in breadth but not in length. M. M. Swann¹⁰² has

* Regions in which molecular orientation or particle orientation occurs are, under appropriate conditions, optically anisotropic (see Lowry¹²⁵).

recently obtained improvements in polarization technique which seem likely to facilitate the analysis of systems of weak birefringence.

The muscle fibre contains myosin—a protein which is threadlike and is known to show birefringence *in vitro* in a flowing stream, due to orientation of micelles parallel to the lines of flow. This protein is generally believed to be responsible for the birefringence of the muscle fibre, being oriented parallel to the fibre axis. The striation of the fibres



TEXT-FIG. 1. Structure of myelin sheath of a nerve fibre.

of striped muscle is believed to be due to alternating regions of orientation and disorientation, the myosin in the isotropic regions being oriented at random, and in the anisotropic regions oriented parallel to the fibre axis (von Müralt¹⁴³). Bernal¹² has suggested that in the apparently isotropic (non-birefringent) region the molecular packing is closer than in the anisotropic region, and the direction of orientation is at an angle to the fibre axis. In contraction the birefringent regions decrease in size and the isotropic regions increase in size. X-ray studies of muscle contraction have not yet proved very fruitful, though some changes have been observed which possibly occur in the myosin molecule during construction (Astbury⁴).

Birefringence has also been observed in other contractile tissues, including cilia and pseudopodia. It is probably true that most protoplasmic movement is associated with changes in the arrangement of long protein molecules. Frey-Wyssling⁶² has recently published a stimulating account of the possible functions of long molecules in relation to the properties of cells.

Schmitt and Bear¹⁶² have shown from the optical properties of the myelin sheath of medullated nerve that the myelin consists of layers of lipid molecules oriented radially. It is probable that the lipid molecules are arranged in concentric cylindrical shells, two molecules thick, separated by layers of protein (Text-fig. 1).

A similar lipid-protein structure is believed to exist in the rods and

cones (Schmidt¹⁶¹), in this case with the sheets of molecules at right-angles to the long axis of the cells. This lipoid-protein structure consists of a repetition of units, each of which is a bimolecular lipoid layer stabilized by adsorbed protein. It was this basic unit which was suggested by Danielli⁴⁷ for the cell membrane. These types of structure found in myelin and the rods and cones may thus arise directly from the cell membrane.

A great advantage in visual microscopy of thin objects can be obtained by use of the phase-contrast microscopes developed by Zernicke¹⁷³ and Burch²⁰. The position in this field has recently been briefly reviewed by Taylor¹⁶⁹. This method reveals differences of refractive index in the object, which with a suitable optical system can be used to cause different degrees of interference in the image, so that sites of different refractive index can be made to appear lighter or darker than the background. The method has been particularly useful in the study of living objects, with which staining methods are of very limited application. The movements of chromosomes in meiosis and mitosis, which previously could be observed only with difficulty, are readily observed with phase-contrast methods.

b. Electron Microscopy

Substantial progress has been made in the development of electron microscopic techniques for biological material. Unfortunately, as water absorbs electrons very strongly, there are great difficulties besetting the examination of living organisms with electron beams, so that all studies have been made on dried specimens. Specimens substantially thicker than 0.1μ absorb electrons so strongly that they cannot be examined by current techniques, so that work has been confined, with few exceptions, to small cells, or to smears, squashes, teased fragments, or special tissue cultures¹⁵². Studies on sections have been few as a result of the difficulty of obtaining sections at 0.1μ or less³⁷.

Amongst the more striking results are studies on flagellae, especially that of Harley Brown⁷⁴ on structure in relation to the mechanism of flagellum action, and those of van Iterson¹⁰⁴ on the structure of flagella. Very stimulating observations have been made on viruses; particularly noteworthy are those on vaccinia by Green, Anderson, and Smadel⁷⁰, which confirm the conclusion drawn from X-ray studies that this virus is a complex body, and on bacteriophage which in some instances appears to consist of a spheroidal 'head' and a relatively long 'tail'. The significance of this structure of phage is unknown. Some of the studies on bacteriophage, such as that of Luria and Anderson¹²⁸, indicate that the 'head' of the phage particle is not homogeneous, and recent studies by Wyckoff¹⁷² on the formation of phage in bacteria show that at an early stage in phage formation the 'head' may apparently be hollow.

Electron microscope studies on chloroplasts reveal a considerable amount of internal structure (Algera, Beyer, van Iterson, Karstens, and Thung²).

A few studies have been made of viruses inside cells in tissue culture. Those of Claude, Porter, and Pickles³⁷ on material contaminated with the Rous sarcoma virus are particularly striking. Particles of the size ascribed to the virus can readily be seen in the cytoplasm of fibroblasts, sometimes as single particles, sometimes as clumps of two or more particles, sometimes arranged in a rod-like form, and sometimes in a crystal-like mosaic. In some specimens the occurrence of doublets is far more frequent than is statistically probable on a purely random distribution—a fact which has led to the speculation that a doublet represents a particle which has recently duplicated itself. The virus has also been observed in white cells from the buffy coat layer of chicken blood. This observation, however, cannot be interpreted as indicating that the spindle cells of the sarcoma are derived from the white cells of the blood: it is just as possible that the white cells are independently infected, or, indeed, that the particles seen have been ingested by the phagocytes and are in process of destruction. In these studies there is the further difficulty that it is by no means certain that the particles seen in the cell and found characteristically in the purified virus are in fact identical with the infective agent. Among the factors which make the identification dubious are (1) the occurrence of particles of a practically identical size in embryo extract and in extracts from tumours which have not yet been shown to give rise to a filterable virus, (2) the minimal infective dose of the large particles which will give rise to a tumour contains about 2,000 particles,²² amongst which a few smaller or modified particles would easily escape notice. Among the points to be considered is the possibility that the large particles are merely a characteristic of rapidly growing chicken tissue, and the possibility that the Rous virus, if to be identified with the particles seen in infected cells, is a mutant of the form found in normal rapidly growing tissue.

A third field of great interest has been the study of natural protein fibres. Contributions to this have been made by many workers, that of F. O. Schmidt and his colleagues being particularly outstanding. The point of greatest interest has been the discovery that many natural fibres, when treated in an appropriate way, can be shown to be banded. This is true of fibrin fibres (Ruska and Wolpers¹⁵⁹), of striated muscle (Schmitt, Hall, and Jakus¹⁶³), smooth muscle fibres (Ardenne and Weber³, Hall, Jakus, and Schmitt⁷³), collagen fibres (Schmitt, Hall, and Jakus¹⁶³), trichocyst sheaths (Jakus¹⁰⁷), and of material from the axoplasm of nerves (de Robertis and Schmitt¹⁵⁶). In the case of striated muscle the bandings are the same as those observed in the microscope. In the case of the other materials the bands are in a sense new, although

the spaces between the bands in some cases correspond fairly precisely with spacings which had previously been discovered by X-ray techniques. The precise significance of these arrangements of bands is not known. It would be surprising if the existence of a type of structure in so many types of natural protein fibres were not of great significance. One possibility is that the existence of the bands, giving rise to an uneven distribution of molecular forces along the length of the fibre, facilitates more precise adlineation and contact between the different identical protein chains which make up each relatively large fibre. This would probably give the fibres much more mechanical strength than would be found with a less precise adlineation. Another possibility is that the bands define the basic units which are synthesized in the cell at one time, and which are later joined together end to end to constitute a fibre.

Callan and Thomas²¹ have recently made a very interesting study of the structure of the nuclear membrane of amphibian oocytes. This has been shown to consist of a thin film about 100 Å in thickness, apparently supported on a relatively coarse meshwork.

We can expect the use of the electron microscope to yield a great deal of further information of value to the biologist. It is possible that the value of electron microscopy will be increased by further developments which seem possible on the technical side. Amongst these are included the use of high velocity electrons, which will permit of the use of thicker specimens than does the present microscope; the development of a polarizing electron microscope would enable studies of molecular orientation to be made in the same way as is possible with the light-polarization microscope, but with the advantages conferred by the greater resolving power of the electron microscope; a proton microscope, the development of which is now being considered, may also have advantages. Then there is the rather difficult problem of the development of electron lenses for studying the distribution of radioactive isotopes in tissue sections, &c. The resolving power possible without the use of electron lenses, i.e. by simple application of the material to the emulsion of a photographic plate, is usually much too small to be of value on the cytological level. An addition of tenfold to the resolving power and magnification would be of immense value. Unfortunately, the electron emission from isotopes has a wide band of velocities, so that a correction analogous to that for chromatic aberration is necessary before an electron lens becomes useful. This problem has not yet been solved.

An interesting suggestion for the use of neutrons in the study of cytochemical distribution of substances has been made by J. E. Harris⁷⁶. He suggests that the material to be studied should be applied to the emulsion of a photographic plate and then exposed to slow neutrons. Under these conditions some elements, of which lithium and boron are

the more notable for the biologist, are readily disintegrated with the emission of particles which give rise to easily recognized tracks in the emulsion.

c. X-rays

Use of X-rays has permitted progress in the study of mutations, chromosome breaks, the size of genes and viruses, and the multiplication of viruses in cells. The use of X-rays for the study of globular and fibrous proteins, though, of course, hardly to be classed as the study of cells, has nevertheless been of much value in enabling us to understand the function of these molecules in cells; in this direction much expansion of results can be anticipated. Lea¹¹⁷ has recently surveyed a large part of the field of the mode of action of radiation on cells. He has laid particular emphasis on the so-called target theory, according to which the action of many forms of radiation is produced by ionization consequent on the passage of radiation through the region containing a target. It is usually assumed as part of the theory that one ionization produced in the target area is sufficient to modify or destroy the target. As typical of the materials to which this theory can be applied are viruses, genes, and chromosomes. By the use of X-rays it is possible to measure the volume of the target, and by the use of alpha rays it is possible to measure the average cross-sectional area of the target. Information obtained in this manner about the size of viruses has been confirmed in many instances by electron microscope studies and by ultra-filtration of viruses. In some instances the size of the target determined by these radiation techniques is smaller than the size determined by other techniques. This suggests that the virus is not homogeneous but contains a volume, damage to which is much more important than is damage elsewhere. In the case of the vaccinia virus such a conclusion was drawn from the discrepancy between the sizes determined by radiation and other techniques, and an indication of the correctness of this conclusion has been given by electron microscope studies, which show that the virus is not homogeneous but contains a region having a different structure to that of the rest of the virus. By both electron microscope and radiation techniques this region appears to be of the order of one per cent. of the total volume of the virus.

The target theory has recently been used with much success in connexion with Sonneborn's¹⁶⁷ studies on cytoplasmic inheritance in *Paramecium aurelia*. Sonneborn showed that certain strains of *Paramecium* emit a substance which kills other strains. This property is exhibited only by those members of the strains which (a) contain a nuclear gene *K*, and also (b) contain a factor not present in the nucleus but only in the cytoplasm, which Sonneborn called kappa. The factor kappa can reproduce itself in the cytoplasm but only if the gene *K* is present in

the nucleus. Consequently kappa was suspected to be a cytoplasmic gene. Studies were made of the size of kappa with radiations, from which it was found that kappa is much larger than the size usually attributed to a gene and, in fact, should be readily visible in the cell. A search was made for particles of this size, using the Feulgen reagent, which was successful. It now appears likely that kappa is in fact a Rickettsial infection of *Paramecium* which can only proliferate in *Paramecium* containing the gene *K*, which is not normally pathogenic to its host, and which in some way promotes the production of a substance which is pathogenic to *Paramecia* not containing kappa.

The production of chromosome breaks by X-rays has permitted many studies of broken chromosomes, chromosome fragments, and of genes transferred to abnormal positions in chromosomes. The size of the gene has also been shown to be of the order of 20–60 Å (using the target theory). This average value is in reasonable agreement with the size of genes determined by other methods. It should be noted that the size of the gene appears to be of the same order of magnitude as that of a small virus.

Although the production of mutations by X-rays has been used for a number of years, it is only comparatively recently that the method of using mutations induced in single genes by radiations has been used to analyse the biochemical detail of the action of genes. Of recent years extensive studies have been instituted of the biochemical effects of the mutation of single genes, using yeasts, moulds, and bacteria¹⁶⁸. A commonly observed phenomenon is that the simpler forms of injury can be compensated for by adding to the medium upon which the organism is growing a single chemical substance, the nature of which depends upon the gene injured. It has therefore been concluded that each gene controls the production of a single substance, which is necessary for the growth of the cell. This view, however, has probably been somewhat exaggerated. It is well known that practically all biologically important molecules can be produced by unicellular organisms. It thus seems extremely difficult to attribute much of the enormously complicated process of the evolution of organic form in animals and plants to these simple chemical factors. For instance, it is very difficult to see how any simple chemical substance could be responsible for an animal having six toes instead of the normal five. It is therefore to be anticipated that the genetic structure of the higher organisms must include genes which have a specific effect upon form.

Very important results have been obtained by irradiating bacteriophage-infected bacteria. Latarget¹¹⁵ found that when *Escherichia coli* infected with one bacteriophage T₂ particle per cell are irradiated during the 21-minute period which ensues between infection and lysis, the following facts are disclosed: (1) for the first seven minutes no

multiplication of phage occurs, and the phage particle remains unaltered; between the 7th and 9th minutes the phage becomes much more resistant to radiation; at the 9th minute multiplication commences rapidly, resulting in a total of up to 250 phage particles per cell by the 13th minute, when multiplication ceases; between the 13th and 21st minutes the resistance to radiation reverts to normal. Luria¹²⁷, using ultraviolet light, has shown that whilst phage can be damaged in a number of different genetically active sites, each of which is necessary for reproduction of phage, yet if a bacterium is infected with, say, two phage particles damaged in *different* sites, reproduction will occur. Since the phage particles are unable to reproduce individually, some curious process of dissociation of the bacteriophage must occur, permitting independent reproduction of each genetically active centre, with subsequent recombination of the centres.

d. Radiations from Isotopes

Isotopes have so far found three important applications in the study of cells. These are: (a) the cytochemical localization of certain substances, (b) metabolic studies, and (c) permeability studies. In the first field the utility of the technique, as has been pointed out above, is greatly restricted by the poor resolving power which is at present possible, and the results obtained, using substances such as radioactive iodine and phosphorus, have been more impressive on the histological level than on the cytological level.

In the field of cell permeability a number of very important points have been settled. For example, it has been shown by Krogh¹¹² that both sodium and potassium permeate relatively readily both into and out of cells. In some instances, such as that of muscle cells, potassium permeates a good deal faster than does sodium. In other instances the rates are more nearly equal. There is an enormous field to be covered which has as yet been hardly touched. As an indication of the somewhat surprising results which can be obtained may be mentioned Cohen's work³⁸ on the uptake of phosphorus by cells in which bacteriophage is reproducing. It would have seemed probable that some of the intracellular phosphorus would be incorporated in the bacteriophage, but, in fact, the phosphorus found in the phage is all derived from the medium subsequent to entry of the phage into the cell.

Of the numerous metabolic studies which have been made only a few can be mentioned. By the use of radioactive iron it has been shown that the life of the mammalian red cell is of the order of 100 days. This figure is in agreement with those obtained recently by other techniques. Studies with carbon, nitrogen, and phosphorus, using heavy and light isotopes as well as radioactive isotopes, have revealed that all the major components of the body are in a state of surprisingly rapid

flux, being continuously broken down and built up¹⁶⁴. The rates at which these processes occur vary somewhat according to the type of compound which is studied. For example, the phosphorus of pentose nucleic acid changes over more frequently than does the phosphorus of desoxypentose nucleic acid. But even in the case of the more slowly changing components of cells, a 50 per cent. turnover occurs in the matter of a few weeks. This reveals that the whole structure of a cell must be regarded as in a state of flux. No matter how fixed the appearance of a cell may be on the microscopical level, we now know that in point of fact every particle is participating in a dynamic equilibrium.

e. Mitogenetic Radiations

Gurwitsch (1932⁷²) (see *Review*, 1935⁶) claims that some tissues emit a special radiation which induces mitosis in other tissues. It was claimed that these radiations were in the ultraviolet around $220\text{ m}\mu$, and the school of Gurwitsch has since claimed that mitogenetic radiation is closely associated with many other forms of cellular activity. The majority of workers in this field have been unable to substantiate Gurwitsch's claims.

f. Spectroscopy

Use of the spectroscope is limited, but where a process involves change in the absorption spectrum of a cell it may be an extremely useful instrument. Important instances of its use are the work of Keilin, Millikan, and Caspersson. The work of Caspersson has been referred to earlier. Keilin's work has included studies of the intra-cellular oxidation and reduction of the cytochrome enzyme system¹¹⁰, and of measurements of the permeation of substances into red cells, recorded by changes in the absorption spectrum of haemoglobin. Interesting studies have also been made on the absorption of dyes by plant cells (Irwin¹⁰³). Millikan has studied the intra-cellular oxidation and reduction of muscle haemoglobin¹³⁸.

At present studies in the infra-red have not been of any great significance. But if a technique can be developed for the spectroscopic examination of the infra-red absorption spectrum of different parts of cells many important results would probably emerge. It is possible that such a technique may be evolved using the reflecting microscope.

g. Microfilms

Films of a number of processes such as the fertilization of eggs have been available for many years. But it is only comparatively recently that full use of the possibilities of these techniques has appeared. Recent studies have included observations on dividing cells with the polarizing and phase-contrast microscopes, and on the feeding mechanisms of ciliates. By slowing these films down, using selected stills, and making

measurements on successive stills, it is possible to analyse in great detail many of the processes involved in such a phenomenon as cell division. It is to be anticipated that studies of this sort will enable us to submit theories of cell division to quantitative tests. Advances in many other fields may be anticipated of importance equal to these studies in cell division.

iii. MECHANICAL INVESTIGATIONS

a. Centrifugal Force

The Cell Membrane. The use of centrifugal force in the study of the cell membrane is discussed in Chapter III.

The Viscosity of Protoplasm. The viscosity of a fluid may be determined by studying displacement of a particle due to Brownian motion, or from the rate of movement of a particle in a centrifugal, gravitational, electrical, or magnetic field. Of these possible methods, centrifugal force and Brownian motion have proved the most satisfactory. Table I shows values for the viscosity of the cytoplasm of a number of different cells. Usually the values obtained are not greater than ten

TABLE I. *The Viscosity of cytoplasm (in centipoises)*

Viscosity of water = 1 centipoise
Viscosity of olive oil = 100 centipoises

<i>Species</i>	<i>From Brownian movement</i>	<i>From centrifugation</i>	<i>Author</i>
Spirogrya . .	2-47	..	Baas-Becking, Bakhuyzen and Hotelling, 1928 ⁵
Arbacia egg .	4	2	Heilbrunn, 1928 ⁸⁹ , 1926 ⁸⁸
Amoeba . .	6	2	Peckarek, 1933 ¹⁵⁵ , Heilbrunn, 1929 ⁹⁰
Sabellaria egg .	20	..	Harris, 1935 ⁷⁵

times that of water, though in exceptional cases values as high as 8,000 have been obtained (Fetter⁶⁰, for *Paramecium*). Where an apparently very high viscosity is obtained, it is probable that intracellular streaming, or intracellular structures, are interfering with the free movement of the particle under observation. The low value obtained for the cytoplasmic viscosity of most cells strongly suggests that the cytoplasm does not have a rigid invisible structure. Gray⁶⁸ found that the nucleoli of the large nucleus of *Asterias* or *Echinus* oocytes move freely through the nucleus under gravitational force, always coming to rest on the 'floor' of the nucleus. From the rate of fall it was calculated that the viscosity of the nucleus was 10 times that of water.* It follows, therefore, that in this case also there can be little in the way of a rigid,

* Heilbrunn⁸⁹, corrected by Harris⁷⁵. Several assumptions, including the difference between the densities of nucleus and nucleolus, are made.

permanent structure extending throughout the nucleus. Harris⁷⁵ also studied the fall of the nucleolus of the *Echinus* oocyte under gravity. He concluded that the nucleus is a homogeneous thixotropic fluid of viscosity, calculated from Brownian movement, *ca.* 10 times that of water. In addition, it was found that there is a force acting on the nucleolus, possibly electrical, which is constant in magnitude and direction for a given oocyte. This force produces secondary movement of the nucleolus in a direction independent of the orientation of the oocyte. The force is abolished by M/500 KCN.

On the other hand, using the centrifuge microscope, Harvey and Marsland⁸⁶ have shown that whereas most of the particles observed move at a uniform rate in a centrifugal field, others show intermittent jerky motion, and it was concluded that the cell is in a state of incipient gelation, and that rapidly reversible sol $\rightleftharpoons$ gel changes are occurring in local regions of the cytoplasm. There is usually a semi-permanently gelled region close to the plasma membrane (the cortex, or ectoplasm).

Studies of the change in cytoplasmic viscosity during cell division have been made, particularly by Heilbrunn⁸⁷ in 1921. There is a rise in viscosity associated with the development of the sperm aster, followed by a fall, then by a second rise associated with development of the amphiaster. These results have been confirmed, qualitatively, by estimates of viscosity made by Chambers²⁴ using the micro-dissection technique. In death most cells set into a gel, and, as Harvey has remarked, the action of stimuli of various kinds is to cause an increase in viscosity, culminating in irreversible gelation if the stimulus is sufficiently strong.

Centrifugal force has been used with much success in the study of the degree of differentiation of the cytoplasm of egg cells. By centrifuging regions of the cytoplasm into abnormal positions, and by use of micro-dissection techniques, it has been shown that ova before fertilization can be separated into two classes. In one class of eggs the cytoplasm appears to be differentiated so that some regions are destined to give rise to definite parts of the embryo. With these eggs marked abnormalities in development occur when cytoplasmic components are placed in the wrong positions. With other eggs displacement of the visible bodies in the cell and of granules, &c., does not affect the polarity of the egg, the cleavage pattern or organ formation. Much of this work was done in the early part of the present century by Boveri¹⁷, Lyon¹²⁹, F. R. Lillie¹¹⁹, Morgan¹⁴²; and Conklin⁴². Most of the studies so far mentioned were made with rather low centrifugal forces. It is not yet clear whether very high centrifugal forces, capable of causing sedimentation of the dissolved proteins of the cytoplasm, will have contrary results. In one instance a negative result was obtained. Beams and King¹¹ centrifuged *Ascaris* eggs for four days at 150,000 $\times$ gravity and found

that they would still develop normally when fertilized. Costello⁴³ has found that if *Arbacia* eggs are centrifuged before fertilization until they finally divide into two halves, one 'light', the other 'heavy', practically no fertilization membrane appears on the 'light' half and a very marked membrane is found on the 'heavy' half. Evidently centrifugal force can shift a substance or substances connected with the elevation of this membrane.

The technique of centrifuging eggs into halves (and then into quarters) has been developed very largely by E. N. Harvey⁸⁴. When centrifuged, stratification occurs, oil droplets moving to the centripetal end of the cell, and heavy granules, &c., to the centrifugal end. The tendency of these strata to move in opposite directions elongates the cell, which spontaneously divides into two when its length is π times its diameter. This is a particularly useful technique. Studies have been made of the respiration of light and heavy halves (Shapiro¹⁶⁵) and of the distribution of enzymes between light and heavy halves (Holter¹⁰⁰). Among the most important discoveries made by this technique is the observation of parthenogenetic merogeny in *Arbacia* egg halves by E. B. Harvey⁸³. Halves without nuclei, when subjected to parthenogenetic reagents, cleave in a relatively normal manner and will continue to do so up to the stage of a blastula of some 500 cells. That nuclei may divide and mitosis occur without cell division has been known for many years. Mrs. Harvey's observation shows that cell division may occur independently of nuclei and that therefore the minimum requirements for cleavage are contained by enucleated cytoplasm.

b. Micro-dissection and Micro-injection

Micro-dissection and micro-injection operations, carried out on single cells, have provided a wealth of valuable information. The two chief difficulties encountered (apart from the development of the necessary manual skill) are that (1) operations on cells instantly make them abnormal, and (2) conclusions reached are of necessity relatively subjective in many cases. The first difficulty is the more serious: when an organ is removed from such an animal as a dog, we have very many ways of estimating the degree of abnormality introduced. When a single cell is operated upon we have as yet only rather crude criteria of abnormality; if cytolysis occurs, or gelation, or permeability to dyes is abnormal, if pigment granules and other cytoplasmic inclusions look abnormal, or if in the development of an egg abnormalities occur, we know serious damage has been done. Beyond this it is hardly possible to go. Thus if mammalian physiology were exposed to the same restrictions as cell physiology, we should have to judge the results of an operation by whether the dog died, was apathetic or normal in movement, whether it passed into rigor or tetanus, whether the disposition of vital stains

was abnormal, whether its hair fell out, and whether its puppies showed gross anatomical abnormalities.

The major contributions to technique have been made by Chambers, Peterfi, and de Fonbrune. A fairly detailed description of results obtained up to 1924 is given by Chambers²⁵.

Many cells are surrounded by a tough elastic pellicle (such as the vitelline membrane of egg cells); this may be dissected away from the cell. For example, a slit may be cut in the pellicle of an *Arbacia* egg, through which the egg may be expressed without apparent damage to the plasma membrane. The surface of such a naked cell is readily wetted by oils (Chambers and Kopac³⁵) and appears to be liquid. It can be torn by a sharp movement of a dissection needle, in which case the cytoplasm usually pours out and mixes with the surrounding fluid; substances normally unable to enter a cell do so readily through such a tear. But a needle moved carefully passes into the interior of the cell without breaking the membrane. The membranes of most cells are relatively tough and have marked elasticity: an erythrocyte may be stretched between two needles to a length several times its normal diameter and will recover its original shape on release.

The interior of the cytoplasm is usually fluid and of low viscosity, except for a cortical layer (the ectoplasm) which is jelly-like in consistency and usually contains few inclusions. There is some evidence that this layer is intimately concerned in cell cleavage: a fragment cut from an *Asterias* egg will develop on fertilization in a relatively normal manner if a sufficient fraction of the cortex is included in the fragment. If there is little or no cortical material the fragment will not cleave even if it contains the egg nucleus. Pseudopodia of amoebae, when pinched off from the main body of the amoeba by a needle, retain the ability to move and to ingest and digest particles for a considerable time. If a small volume of water or salt solution is injected into an amoeba it usually appears to mix readily with the cytoplasm and the excess water is eventually expelled through the cell membrane. Otherwise local liquefaction of the cytoplasm may occur and the liquefied area frequently rounds up and then resembles a vacuole. If a large amount of water is injected the amoeba may be killed. If it eventually recovers, a large part of the injected fluid may be pinched off in a blister. Basic dyes injected cause local coagulation of the cytoplasm, the coagulum eventually becoming dispersed through the more fluid cytoplasm as coloured granules. Acid dyes frequently cause liquefaction of the cytoplasm and the liquefied area may be pinched off.

The mitochondria of marine eggs appear, when in the filamentous form, to be relatively solid, sticky, elastic bodies. The pH of the fluid cytoplasm of most cells is about 7, as given by injected acidic indicators (which are likely to be more reliable than basic indicators) and the

nucleus, where its pH can be measured, is usually 0.5–0.6 units more alkaline.* Similar results have been obtained with vital stains. Studies on oxidation-reduction potentials were first made by Needham and Needham¹⁴⁵, and have been extended by Chambers and his colleagues.

The nucleus shows quite different properties according to whether it is in the mitotic or intermitotic condition. The resting nucleus appears in most cases to be liquid. Tearing the nuclear membrane causes disintegration of the nucleoli followed by liquefaction of the cytoplasm and often by cytolysis. The liquefaction of the cytoplasm spreads from the nucleus outwards. When transposed from the interior to the exterior of a cell through a tear in the plasma membrane the nucleus may swell to bursting or set to a rubbery gel.

According to Chambers, the active nucleus varies in its behaviour according to the stage of development attained. When the very early prophase nucleus of grasshopper spermatocytes is injured, filaments appear in the nucleus composed of granules adhering to an elastic hyaline thread. These shorten and thicken, with apparent fusion of the granules, into rods reminiscent of chromosomes. One of the nucleoli also gives rise to a similar body. If injured at a slightly later stage, typical prophase chromosomes appear rapidly, and if prophase chromosomes are already visible inside the nucleus, pricking the membrane causes an increase in definition and transition to metaphase chromosomes. Individual chromosomes may be pulled away from the spindle during mitosis. Such detached chromosomes are elastic bodies with a cortex apparently different from the hyaline central core.

The spindle varies in viscosity and gel-like properties, both with time and with species. It may be a stiff gel, or relatively fluid. The amphister may have sufficient rigidity to distort the shape of the cell (Gray⁶⁷). The sperm aster of starfish and sea-urchin eggs is gelled, but the centrosphere (in the centre of the aster) is fluid: the gel is most rigid close to the centrosphere and becomes weaker farther away from the centrosphere (Chambers). Stirring the interior of the cell may cause the amphister to disappear, but it will reappear on cessation of stirring if the agitation is not excessive. In nuclear division the two asters are firmly united by the spindle until elongation and division of the spindle occurs, after which the asters are readily moved independently.

Thus micro-dissection shows up two main features of cell structure: (1) the role of the membranes, (2) the texture of the cell constituents. A certain amount of light is also thrown on many other features of cell physiology.

It will have been observed that the parts of the protoplasm concerned

* It is possible that this difference is fictitious and is entirely due to the protein error being different in the two cases, for the cytoplasm and nucleus contain quite different proteins.

in movement are capable of gelation and are elastic. We have previously noticed that the same structures are also optically anisotropic. We shall later discuss the significance of these properties.

Some very stimulating results have been obtained by Commandon and de Fonbrune⁴¹ with enucleated amoebae. If a nucleus from another amoeba is pushed into the enucleated amoeba at any time up to 48 hours after enucleation, the enucleated amoeba will recover completely and will proceed to divide and otherwise behave normally. It is necessary, however, that the nucleus which is being moved from one amoeba to another shall not come into contact with the environmental fluid. If it does come into contact with the environmental fluid, it is rejected by any amoeba into which it is inserted.

An important recent development is the use of the Cartesian Diver for micro-metabolism experiments (Linderstrøm-Lang¹²³; Linderstrøm-Lang and Glick¹²⁴). By this means the study of single cells or of groups of small cells removed from, for example, an invaginating blastula by micro-dissection, is much facilitated. Needham and his colleagues¹⁴⁶ have made an extensive study of metabolism in relation to morphogenesis using this technique.

c. Pressure

Reducing the pressure on an organism below atmospheric pressure has little effect until asphyxiation commences. Increasing the pressure above a limit critical for a given organism at a given time has the effect of liquefying the plasma gel, and so causes most profound changes in the behaviour of cells. The physico-chemical basis of this liquefaction is not clear and we shall therefore not discuss it. The elucidation of the mechanism of such liquefactions is of outstanding importance.*

The general result of liquefaction of cytoplasmic gels is the interruption or suppression of all forms of behaviour involving active protoplasmic movement, and sufficiently high pressures may cause death, possibly due to denaturation of proteins, or to the disintegration of essential organized systems. The lower pressures sometimes cause enhancement of activity.

Grundfest and Cattell⁷¹ found that with single nerve fibres pressures between 300 and 500 atmospheres increased the height of the action potential by 10 per cent. and the duration by 20 per cent. As the pressure is raised towards 1,000 atmospheres the potential falls off, the rate of conduction slows, and finally the response disappears. All the changes are reversible.

* It is possible that liquefaction is due to increased hydration of protein molecules; but as Cattell²³ remarks, 'It would be difficult to eliminate completely the possibility that the changes in viscosity are secondary to changes resulting from stimulation or chemical reactions.'

With muscle (see Review, Cattell²³) the sudden application of 300–400 atmospheres to a resting muscle causes a small contracture. There are three apparently distinct effects on the behaviour of electrically stimulated muscle: (1) relatively low pressures cause the liberation of more energy, shown by an increase in the initial heat production and an increment in the tension of twitch; (2) at higher pressures the response is both slower and weakened, suggesting a decrease in elasticity and possibly an increase in viscosity also; (3) finally, at a sufficiently high pressure (c. 1,000 atmospheres), contraction is abolished. If in phase (2) the pressure is suddenly released during the development of a twitch, the twitch is augmented above the value which would have been obtained if the whole operation had been carried out at atmospheric pressure: this suggests that the increase in mobilization of energy found at the lower pressures persists at least during the second phase. The mobilization of this energy is complete in the first eighth of the contraction process (Brown¹⁹).

When frog chromatophores under contraction due to adrenaline are subjected to pressure, the chromatophore relaxes to a degree which is roughly proportional to the pressure which is applied.

Marsland and Brown¹³⁵ found that 400–600 atmospheres was sufficient to cause liquefaction of the plasma gel of *Amoeba proteus* and *Amoeba dubia* with consequent retraction of pseudopodia and rounding up into a roughly spherical shape. Kitching and Pease¹¹¹, however, found that the very long tentacles of the suctorian protozoan *Ephelota coronata* may resist 800 atmospheres without liquefaction. As mentioned previously, structures with liquid surfaces and no rigidity spontaneously divide when their length exceeds π times their diameter: thus the tentacles of *Ephelota* would break into a number of small droplets if liquid, and are only prevented from doing so by the rigidity of the plasma gel. When the plasma gel liquefies under high pressure this rigidity is removed and the tentacle begins to retract, but then divides spontaneously into minute spherical droplets. According to Marsland and Brown¹³⁵, pressures up to 160 atmospheres cause a decrease in thickness and an increase in length of pseudopodia, and the sudden application of 250 atmospheres will cause an abrupt cessation of pseudopodial activity, but not retraction. From the ease with which internal granules may be centrifuged down it was inferred that there is a continuous decrease in protoplasmic viscosity with increase in pressure. As Marsland and Brown indicate, such movements of granules are not necessarily accurate indications of relative protoplasmic viscosity, since there are many complicating factors present.

Sufficient pressure causes retraction of the cleavage furrow in developing eggs. With two species of *Arbacia* Marsland¹³⁴ found that no furrow will form at pressures greater than 350 atmospheres, and that previously

formed furrows recede at this pressure. At lower pressures the rate of impingement of the cleavage furrow on the division axis is reduced. On the other hand, a pressure of more than 800 atmospheres is required to block cleavage of the eggs of *Ascaris*.

Protoplasmic streaming velocity and viscosity are reduced simultaneously by pressure. With *Elodea canadensis* streaming is abolished at 400–500 atmospheres.

Marsland has found that for each increment of 68 atmospheres in pressure a number of cell characteristics are diminished to 0.76 of their former value. This is true of the viscosity of eggs of two species of *Arbacia*, of two species of amoeba and of the leaf cells of *Elodea*, and also the rate of impingement of the cleavage furrow on the division axis of the two species of *Arbacia* and of the velocity of protoplasmic streaming in *Elodea*.

From these studies relatively detailed hypotheses have arisen concerning the role of reversible gelation processes in cell division, protoplasmic streaming, and amoeboid movement.

Clearly there must be considerable complication in the mechanisms underlying these changes produced by pressure. It is just possible, however, that the factor underlying inhibition of movement is in all cases the same—increasing hydration of the protein molecules involved. There is some evidence in the literature that contraction, both of striped and of plain muscle, and the cleavage of cells, is associated with a decrease in solubility of long protein molecules (Mirsky^{139,140,141}; Bate-Smith⁷; Danielli⁴⁹). According to Mirsky (1938), this change may also be produced *in vitro* by dehydration. We must therefore consider the possibility that one of the primary actions of high pressure is to prevent this dehydration and thus prevent movement. If this general line of argument is correct, it follows that proteins of animals living in the ocean depths must be adapted to considerable pressures, and thus differ in some way from the proteins of animals adapted to low pressures.

These studies of the effect of pressure place a most important instrument in the hands of biologists. It will be of great interest to see, for example, whether cultured kidney tubules retain their secretory power under high pressure, and to see whether stomach tissue secretes HCl under high pressure; light may be thrown on other problems, such as the secretion of saliva by the salivary glands and the nature of the forces involved in the invagination of the blastula.

d. The Measurement of Volume Changes

The study of volume changes has been useful mainly for permeability studies, though in other fields this method is sometimes a useful adjunct to other methods. Before about 1920 the most important part of the

work on permeability was carried out by botanists, using the plasmolysis technique. The volume of a cell is a function of the number of osmotically active particles within it, except in cases where increase in volume is restricted by the rigidity of an enveloping membrane, as is the case with many bacterial and plant cells. Change in the number of intracellular particles (ions, sugar molecules, colloids, &c.) brings about a change in the volume. The number of particles within the cell may be modified by passage of molecules through the cell membrane or by the formation or destruction of molecules inside the cell; changes in the state of aggregation of intracellular molecules may also be important. The osmotic pressure of the external medium may or may not be important according to the equilibrium conditions of the cell.

Under the conditions in which plant cells are usually studied, they are prevented from attaining osmotic equilibrium with their environment by their external membrane. If they are placed in a solution with a higher osmotic pressure than the plant cell, there is a tendency for water to be withdrawn, so that unless the substances in the environment penetrate rapidly the cell shrinks, its plasma membrane is withdrawn from the cell membrane, and the volume of the cell is only restored as a result of penetration of osmotically active molecules from the environment. The rate at which return to the initial volume occurs is a function of the rate of penetration of osmotically active molecules, and consequently permeability constants may be calculated from observations of the rate of volume change. This apparently simple behaviour is often complicated by special permeability characteristics of the membrane.

In the case of the red blood cell the membrane is relatively impermeable to cations and relatively permeable to anions. Such characteristics may lead to volume changes occurring when the cells are transferred from one isotonic solution to another, if the solutions concerned contain ions of different valency types. For example, in NaCl solution the interior of the red cell contains a considerable amount of chloride ion. If the NaCl of the environment is replaced by an osmotic equivalent of Na_2SO_4 , chloride ions exchange for sulphate ions across the cell membrane. At equilibrium, for each two chloride ions originally present there remains only one sulphate ion inside the cell, i.e. the number of osmotically active particles inside the cell is diminished: consequently the red cell shrinks on transference from sodium chloride to sodium sulphate. Furthermore, since the red cell membrane has inappreciable structural rigidity and the cell has no active water-excretion mechanism, the red cell body is a direct function of the external osmotic pressure; as this is lowered the cell volume increases until the cell bursts. Ponder has shown that the volume changes are accompanied by a change to

the discoid from the spherical form, and that the surface area of the cell is unchanged during the swelling process. The light-transmitting power of a suspension of red cells is a function of the cell volume and so may be studied with a photo-electric cell or any equivalent device. Suitable apparatus for accurate work along these lines has been described by Jacobs^{105,106} and by Örskov¹⁵⁰.

The *Arbacia* egg, on the other hand, is relatively impermeable to crystalline anions, and undergoes little change in volume on transferring from sodium chloride to isotonic sodium sulphate. But it does swell in media of lower osmotic pressure. With this cell the surface area increases as the cell swells. Rupture of the surface occurs in solutions having an osmotic pressure less than about 40 per cent. of that of seawater. Similar swelling of both red cells and of *Arbacia* eggs occurs when the cells are transferred to a solution of a penetrating non-electrolyte such as glycol. Many studies on *Arbacia* eggs have been made, especially by R. S. Lillie¹²¹, by Lucké¹²⁶, and by Jacobs¹⁰⁶, and by their colleagues.

Very different is the behaviour of the trout egg (Gray⁶⁷). The volume of this cell does not change with moderate variations in the salt content of the external medium, so long as the cell is alive. When the cell dies, volume changes occur rather similar to those exhibited by a block of gelatine, as a result of the membrane becoming freely permeable to salts and water but not to the yolk globulins, &c.

In the case of muscle cells the volume changes are strikingly dependent upon the nature of the salts in the environment. To a first approximation, the muscle cell membrane behaves as though it were permeable to hydrogen ion, potassium ion, and anions, but not to sodium ion. Conway has given this theory quantitative treatment, and has shown that the volume changes, ionic content, and resting potential of muscle may be calculated with a remarkable degree of accuracy. More recently it has been shown, by Krogh¹¹² in the case of muscle cells and by Maizels¹³¹ in the case of red cells, that active processes are concerned in the movements of sodium, and probably also of potassium across the cell membrane. With both muscle cells and red cells there appears to be an active excretion of sodium ions. The mechanism of this excretion is at present obscure.

As a result of activity the osmotic pressure inside the muscle increases and swelling takes place, followed by other volume changes. Meyerhof¹³⁶ has endeavoured to correlate these changes with the chemical changes occurring during activity.

Swelling methods may also be applied to more complex organisms, e.g. Pantin's work on *Gunda ulvae*¹⁵³. Pantin found that this worm may swell to a remarkable extent without observable injury, provided a trace of calcium is present in the medium.

iv. HEAT AND ELECTRICAL INVESTIGATIONS

a. Measurements of Heat Production

Accurate measurements of the heat production of muscle and nerve have been made by Hartree, Hill, and their collaborators. Many methods of measuring heat production are theoretically available, but of these the shielded thermopyle method developed by Hill and Downing provides the best technique yet developed.

The resting heat rate, both of muscle and of nerve, is practically constant.

The earlier measurements of Hartree and Hill⁸² show that the heat evolution from a stimulated muscle occurs in three phases. The first phase involves a sudden evolution of heat occurring very shortly after stimulation and complete before the maximum tension is reached. The second phase of heat evolution is closely associated with relaxation. The first and second phases may be complete in a fraction of a second. The third phase, which may last for many minutes, is probably due to the chemical processes associated with recovery and is not directly related to the contractile process.

Fenn⁵⁹ found that in an isometric contraction (response to stimulation without shortening) less energy is evolved than in a contraction in which the muscle does work by shortening against a load; i.e. in the latter case the heat evolved + the mechanical work done is greater than the heat evolved in the former case, when no work was done. This appeared to imply that a muscle 'knew' what load it was shortening against, and provided additional energy to compensate for it. Hartree and Hill⁸² found that in a single twitch the total energy set free is the same whether work is done or not; when mechanical work is done, the relaxation heat is diminished by an amount equal to the work. But, in agreement with Fenn, it was found that in a tetanic contraction extra energy is liberated if mechanical work is done.

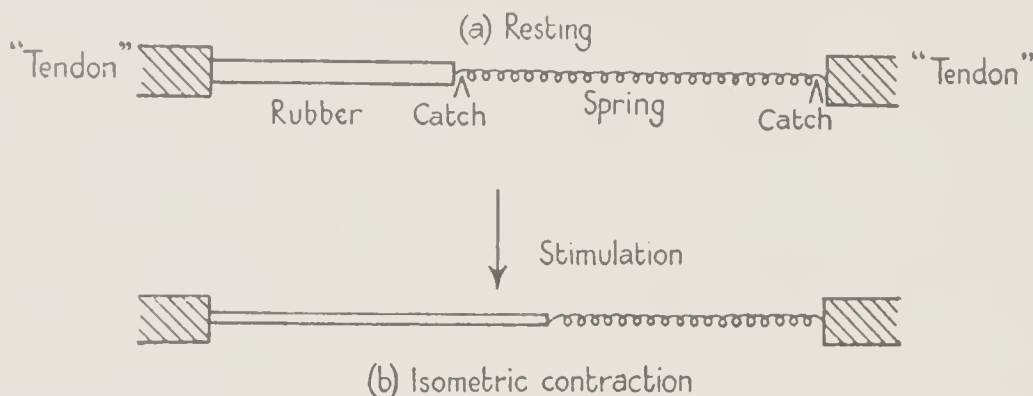
More recently Hill⁹³ has shown that the ratio, total energy released: initial energy is close to 2. For individual muscle preparations the ratio varied between 1.73 and 2.34. The value of the ratio for a given preparation was the same for either single contractions followed to complete recovery or in a long series of contractions in a steady state. During a steady state accumulation of the recovery heat occurs. During such a steady state the rate of recovery heat production may be several times the resting heat production. The ratio, mean heat rate during steady recovery: resting heat rate, varies in individual preparations from 3.8 to 7, i.e. the recovery heat rate lies between 2.8 and 6 times the resting heat rate.

A contracting muscle obeys the extremely simple relationship

$$(P+a)V = b(P_0 - P) \quad (1)$$

or
$$(P+a)(V+b) = b(P_0+a) = \text{a constant.} \quad (2)$$

Here P = load lifted, P_0 = full isometric tension, V = velocity of shortening, b defines the absolute rate of energy liberation, and a is a term having the dimensions of force and defined by the relationship: extra heat* liberated during shortening = ax where x = shortening (Hill⁹²). An active muscle may be regarded as a two-component system, consisting of (i) an undamped, purely elastic element, in series with (ii)



TEXT-FIG. 2. Diagram of muscle fibre (a) resting, (b) in isometric contraction.

a contractile element having the second equation given above as its characteristic equation. According to Hill there may also be viscous and viscous-elastic elements present, but in the case of striped frog's muscle they are relatively unimportant. A 'resting' frog's muscle may be regarded, to a first approximation, as behaving like a strip of rubber (element 1) tied to the end of a spring (element 2) which is stretched between two catches (Text-fig. 2). Stimulation releases the catches, enabling the spring to contract, then in, for example, an isometric contraction, the rubber stretches as the spring contracts. In relaxation an unknown element is brought into action, stretching the 'spring' and allowing the 'rubber' to return to its original length. During contraction the contractile element liberates heat which is contributed to the first heat production, and during relaxation the shortening of the elastic element is accompanied by the conversion of its potential energy into heat which is contributed to the relaxation heat.

The heat production of nerve in response to stimulation is very much less than that of muscle and occurs in two phases: an initial phase, accompanying the response to a stimulus, and a second phase of long duration, probably connected with the chemical events of recovery in which the heat evolution is ten or more times greater than the initial heat. For the non-medullated limb nerve of the crab the initial heat is 2×10^{-2} ergs per sq. cm. of nerve axon surface: for the medullated nerve of the frog it lies between 5×10^{-3} and 2.5×10^{-4} ergs per sq. cm., according to the experimental conditions (Hill⁹¹). As Hill has pointed

* i.e. heat liberated over and above that liberated in isometric contraction.

out, this is a remarkably small amount of energy for a surface phenomenon; for example, the free surface energy of the olive-oil-water interface is about 20 ergs per sq. cm. and of cell surfaces is about 0.05–1.0 ergs per sq. cm. It is therefore very improbable that passage of the impulse involves more than a small physical change in the nerve plasma membrane. Complete breakdown of the membrane is very unlikely. Danielli⁵³ has suggested a theory of conduction of the impulse based on changes of state of the lipoid elements of the membrane, which is compatible with the energies obtained by Hill for the initial heat.

The heat production of the eggs of *Arbacia punctulata* has been studied by Rogers and Cole¹⁵⁷. The heat production of the resting unfertilized eggs is comparatively small. On fertilization the rate of heat production leaps up to a value as much as twelve times greater than the previous rate. This increase is only partially sustained. The rate falls to about two-thirds of this value (6.8 times the original value) after 20 minutes and remains constant for the next 30 minutes. A further fall then occurs to a value 5–6 times the original value and this remains constant up to the eight-cell stage.

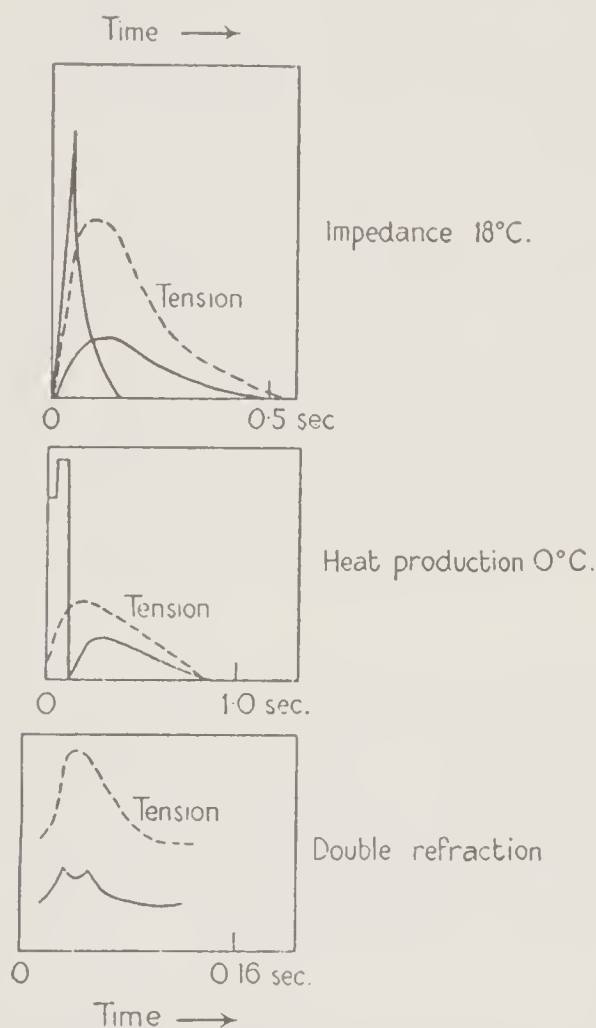
b. Electrical Resistance and Capacity (Impedance) Studies

By the use of alternating current of variable frequency the apparent resistance of biological systems can be obtained. Outstanding work is that of Höber^{96,97} on the red cell, of Fricke and his colleagues on the red cell⁶³, yeast cell⁶⁴, and leucocytes⁶⁵, and of Cole and his colleagues³⁹ on marine ova, the plant cell *Nitella*, and on the giant nerve axons of the squid.

A general conclusion emerges from the work on the various cells; the cell plasma membrane has a capacity of the order of 1 microfarad per sq. cm. and a resistance of the order of 10^4 ohms per sq. cm. The capacity is very high indeed and the resistance, being that of a membrane only 10^{-6} – 10^{-7} cm. in thickness, corresponds to a specific resistance of 10^{10} – 10^{11} ohms, a value similar to that of olive oil. It is remarkable that the wide variety of cells studied should all conform to this pattern. The action of various ions on the resistance and capacity has as yet been studied but little, but Blinks^{15,16} has shown that KCl greatly lowers the resistance of *Nitella* membranes. On the other hand, neither KCl nor CaCl_2 has much effect upon the resistance of the medullated sheath of frog nerve (Danielli⁵¹). There is little doubt that the chief function of the sheath is to act as an electrical insulator, so that it is advantageous to have the resistance of this membrane insensitive to ions.

A further general conclusion is that the higher resistance of cells to direct current is almost entirely due to the cell membranes. By using high frequency alternating current it was found that the resistance of

the interior of, for example, marine eggs in sea-water is comparatively low, rarely greater than four times that of sea-water, although the cells may be highly resistant to direct current. Similarly the resistance of the cytoplasm of the cells of mammals and other land animals is not



TEXT-FIG. 3. Physical changes taking place in stimulated muscle.

vastly different from that of the same animals' plasma. Generally speaking, in other respects the results of resistance and capacity measurements have so far been disappointing, as the method seems to be insensitive to the minor changes in membrane properties accompanying normal cell activity, and in some cases, where membranes are known by other methods to be grossly damaged, no change in resistance or capacity occurs as a result of the damage. Some important results have been obtained on eggs, muscle, and nerve. Changes in the resistance and capacity of some marine eggs occur as a result of fertilization. Hubbard and Rothschild¹⁰¹ have found peculiar rhythmical changes in the impedance of trout eggs, the significance of which is obscure. Dubuisson⁵⁷ has made a successful study of impedance changes in frog muscle. In a strictly isometric muscle there are two waves of increase in impedance

following the stimulus. The first of these probably begins before the contraction and attains its maximum during the phase of rise in tension. The second reaches a maximum during relaxation and returns rather slowly to the base-line; this second change in impedance remains permanently in muscles poisoned with iodoacetate. Dubuisson suggests that the first change is related to the transformations of adenosine triphosphate and the second change to the transformations of creatine phosphate. Text-fig. 3 shows some of the physical changes known to take place in a stimulated muscle. The experiments for the different measurements were made at different temperatures, so that comparison must be made with reference to similar points on the tension curve. If this is done it will be seen that the two phases of the initial heat production and the two phases of diminution of double refraction occur at roughly the same points on the tension curve. We have already referred to the fact that the detailed studies of Meyerhof and his col-

leagues have shown that muscle volume diminishes during contraction. Von Muralt¹⁴⁴ also found that the translucency of muscle diminishes during contraction. Dubuisson⁵⁸ discusses the parallellism between the variation of these physical properties in tetanized muscle and in muscle under anaerobic conditions or poisoned with iodoacetate.

Perhaps the most important work so far accomplished by impedance measurement is that of Curtis and Cole on the change accompanying the conduction of the impulse in nerve and large plant cells. R. S. Lillie¹²² has summarized and discussed the evidence for the view, put forward in 1909 and 1912 by Bernstein¹⁴, that the basic phenomenon in response to a stimulus is a transient increase in permeability to ions, and in the case of nerve the transmission of the impulse consists essentially in the travelling of this increase in permeability along the axon membrane, together with the concomitant discharge of ions through the permeable area which is reflected in the action potential. Using a very refined technique Curtis and Cole^{45,46} were able to show that with *Nitella* and the giant axons of the squid this transient increase in permeability (or conductivity) does in fact accompany the action potential. The conductivity of the plasma membrane is increased about 200-fold. The capacity changes comparatively little.

Small though the change in conductivity is, the residual resistance of the nerve membrane is still comparatively high, so that it is evident that the membrane does not break down during the transmission of the impulse. This confirms the view of A. V. Hill, based on heat-production measurements, that complete breakdown is improbable.

Studies by Cole⁴⁰ have shown that if the impedance-time relations are to be interpreted in terms of circuit theory, a nerve must be regarded as containing an element of inductance. This is a highly improbable occurrence, and it is likely that the apparent inductive element is produced by changes in structure with time, along the lines suggested by Danielli.

c. Potential Measurements

A great variety of cells and tissues have been used for potential measurements, and such studies have played a vital role in several branches of physiology, including the investigation of the transmission of the nerve impulse. The results of these investigations are too well-known to need recapitulation. We may, however, consider for a moment the interpretation of potential measurements. On this subject Gasser⁶⁶ remarked: 'It would be to the advantage of every electrophysiologist to nail this notice on an imaginary laboratory door—*You cannot determine a process from a potential.*' There has been no change in the accuracy of this remark. Students of electrophysiology are therefore obliged to undertake other physical measurements running parallel to potential

measurements, but this work is still in its infancy. Consequently, little more can be said of the resting potential than that it behaves as though it were a potassium diffusion potential arising across the plasma membrane, and that the plasma membrane is more permeable to potassium than to other cations such as sodium and calcium. The potential is to some extent a function of metabolism, but probably rather indirectly so. These conclusions are based on the work of Macdonald¹³⁰, Höber^{95,96}, Osterhout¹⁵¹, Cowan⁴⁴, and many others. The biological studies and the mechanisms by which potentials arise have been reviewed by Davson and Danielli⁵⁶.

The action potential of nerve, muscle, and of large plant cells is now believed by almost all workers to involve a discharge of the resting potential as the result of increased permeability of the plasma membrane. As mentioned before, there is a large transient increase in permeability now known to occur parallel to the transmission of the impulse in *Nitella* and in squid nerve. However, Cole has shown that the magnitude of the impulse is greater than that of the resting potential, so that discharge of the resting potential is not in itself sufficient to account entirely for the action potential. From the work of Katz¹⁰⁸ and of Hodgkin⁹⁹ it is known that in electrical stimulation of nerve, whereas very weak stimuli cause no local response, stimuli a little below threshold do cause a local response, so that the change in permeability is probably capable of gradation and is not necessarily explosive (all or none) in character.

Satisfactory analysis of more complicated systems, such as frog skin, has only just begun.

d. Electrophoresis Measurements

By measuring the rate at which a cell moves in a potential gradient it is possible to calculate the average charge density—that is, the number of ions per sq. cm.—on the cell surface or on the surface of the outermost membrane of the cell. In solutions more dilute than about 0.1 N–0.001 N according to the cell, the results obtained are unreliable: the charge on the cell surface causes the local concentration of ions near the surface to be much greater than the average in the surrounding bulk solution, and in consequence of this the cell is exposed to a lower potential gradient than the average value given by the recording instruments. Consequently many of the results in the literature showing a dependence of surface charge on salt concentration must be regarded with suspicion.

Hartley and Roe⁷⁸ have shown that the concentration of ions near a surface may be determined from the electrophoretic mobility, and Danielli^{48,52,55} has shown that this concentration may be calculated also from the charge density, using the theory of Debye and Hückel to calculate the volume in which the surface ions are distributed. The

concentration of ions near a surface may be several hundred times that in the surrounding bulk salt solution. The average concentration of univalent ions at the surface of cells is usually 1.3–5 times greater; this involves, for example, a pH difference between the salt solution and the cell surface of between 0.1 and 0.7 pH units. It is possible that in localized regions of the cell surface the excess of ions is much greater.

Calculations show that when cells are present in a medium containing a sodium:calcium ratio of the order of 100:1, the ratio of ions at the cell surface is markedly different, being of the order of 1:1. It is probable that the physiologically balanced ratio of 100 sodium ions to 1 calcium ion, which is so desirable for physiological salt solutions, is determined by the necessity of having a physio-chemically balanced ratio of the order of 1 sodium:1 calcium ion at the cell surface.

Abramson's book¹, *Electrophoretic Phenomena*, contains a survey of measurements made on cells.

e. pH Measurements

pH measurements made by vital staining dyes and by the injection of dyes into cells have already been referred to under micro-dissection. Three methods have been used to study pH changes caused by cellular activity. (1) The evolution of CO₂. This is complicated by diffusion factors so that it cannot be used for studying the rapid changes in pH which occur during, for example, the contraction of a muscle, but it is one of the best methods for studying slow processes, such as recovery in muscle. It has been used successfully by D. K. Hill⁹⁴ to investigate the recovery of frog muscle after stimulation. (2) Colour changes with indicators. This is relatively insensitive and open to experimental error, unless spectrophotometric studies can be made. Margaria and Pulcher¹³² used the method for studying changes in muscle. The technique would probably pay for further development. (3) The glass electrode. This method has been used particularly successfully by Dubuisson. It is not available for studying recovery processes, but does enable rapid changes to be studied with some accuracy, subject to an unknown error due to the time taken for acid generated within the cells to reach equilibrium with the fluid bathing the cells. According to Dubuisson⁵⁸ there are four changes in pH taking place in smooth muscle during the contraction-relaxation cycle, and probably the same changes also take place in striped muscle, though the greater speed of the contraction makes it difficult to secure adequate results for striated muscle. These changes in smooth muscle are:

(a) Alkaline; probably pre-contractile; of the order of 1 mm.³ or less of CO₂ per gm. of muscle per twitch; cause unknown.

(b) Acid; probably occurs early in the contractile process; of the order

of 3 mm.³ of CO₂ per gm.; cause, possibly hydrolysis of adenosine triphosphate.

(c) Alkaline; has its maximum at the beginning of relaxation; of the order of 1 mm.³ of CO₂ per gm.; cause, possibly hydrolysis of creatine phosphate.

(d) Acid; begins at maximum tension, but only reaches a maximum several minutes later (at 20° C.); cause, probably lactic acid formation.

Dubuisson, assuming that the pH changes (b), (c), and (d) have the chemical basis given above, has calculated the form of the heat production of smooth muscle, assuming it to be exclusively due to the three reactions mentioned. The anaerobic heat should begin with a positive heat in the early stages of contraction, followed by a second positive heat production near the maximum and during relaxation (early stages), and by a negative heat in the later stages of relaxation. Finally, there is a prolonged positive recovery heat, lasting many minutes, due to the lactic acid formation. This calculated anaerobic heat resembles in many ways the anaerobic heat production of muscle found by Hartree. Dubuisson makes it clear that these theories must be regarded with reserve.

D. K. Hill⁹⁴, measuring the volume of CO₂ produced at 0° C. by the frog sartorius muscle, finds that, under circumstances in which lactic acid is not formed, only alkaline changes associated with the hydrolysis and resynthesis of creatine phosphate are observed. When lactic acid production is permitted, an acidity is found which may (e.g. with cyanide poisoning) take 15 minutes to become half complete. There are interesting variations in these pH changes when enzyme poisons such as iodoacetate, cyanide, and azide are used, which are assisting greatly in allocating the relative positions in time of the various reactions which are known to accompany or succeed contraction.

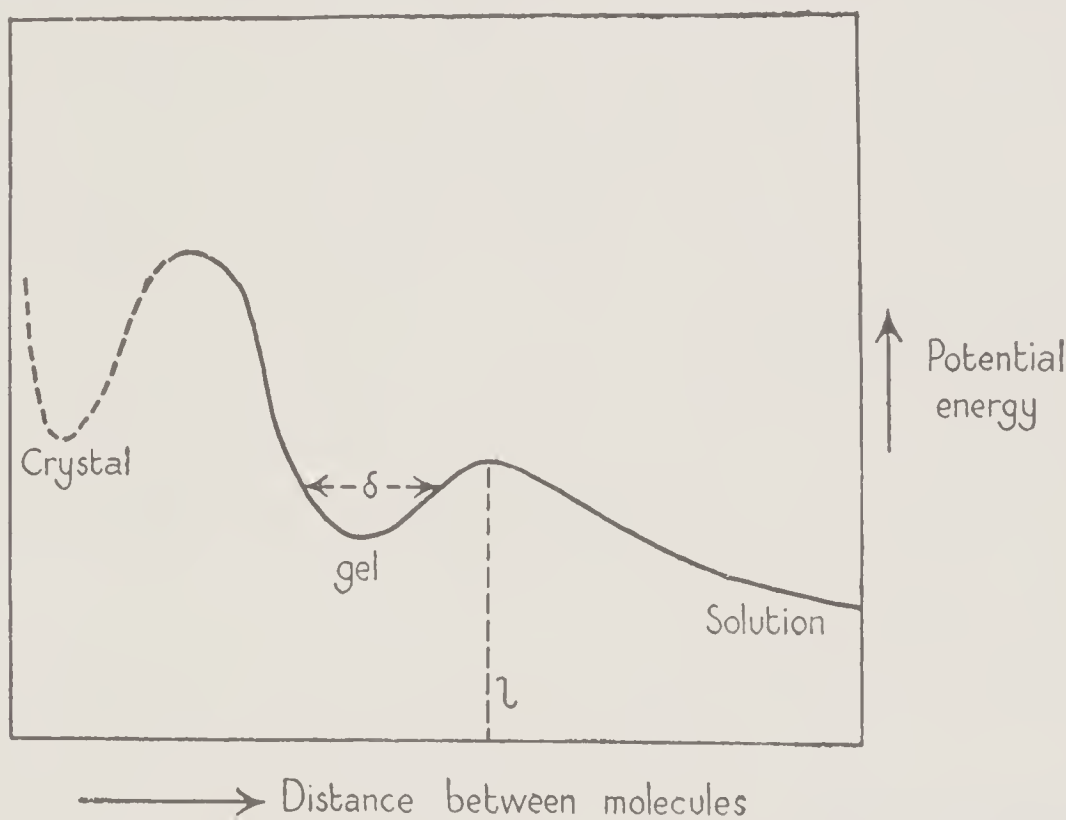
V. MODELS OF BIOLOGICAL SYSTEMS

Owing to the great complexity of living systems, those working on physical properties of cells must frequently resort to a more or less crude model. Until recently these have been largely composed of iron wire, gelatine, nitrocellulose, and olive oil emulsions. Recently, more suitable models have become available as the result of the development of surface and colloid chemistry. These models enable us to test the feasibility of some theories of biological mechanisms with materials which, though not identical with natural membranes and colloids, nevertheless very closely resemble them in many important features, such as the nature of the molecules involved and the physical dimensions of the structures considered. Models which have been particularly important are tactoids as a model of plasma gel, and lipo-protein films and monolayers as

models of the plasma membrane and other biologically important surfaces.

a. Tactoids

If a solution of tobacco mosaic virus is left standing under suitable conditions it separates into two or three components: (a) crystals of the



TEXT-FIG. 4. Potential energy of colloidal particle as a function of the distance between adjacent particles (tactoid forming type of particle).

virus; (b) a top layer which has no rigidity, but which shows double refraction on being made to flow; (c) a denser, lower thixotropic gel layer having some rigidity and which is spontaneously doubly refracting. During the separation of the two layers needle- or spindle-shaped bodies of higher or lower density form in the body of the solution and rise or fall slowly according to their density. Coalescence of these bodies gives rise to the two layers. Bernal and Fankuchen¹³, and Bawden *et al.*⁸, have shown by X-ray studies that the lower layer consists of an hexagonal lattice in which the needle-shaped protein particles are oriented parallel to one another, the distance between adjacent particles varying from 50 to 12.5 m μ as the concentration of protein increases. In the crystal the molecules are much closer together than this.

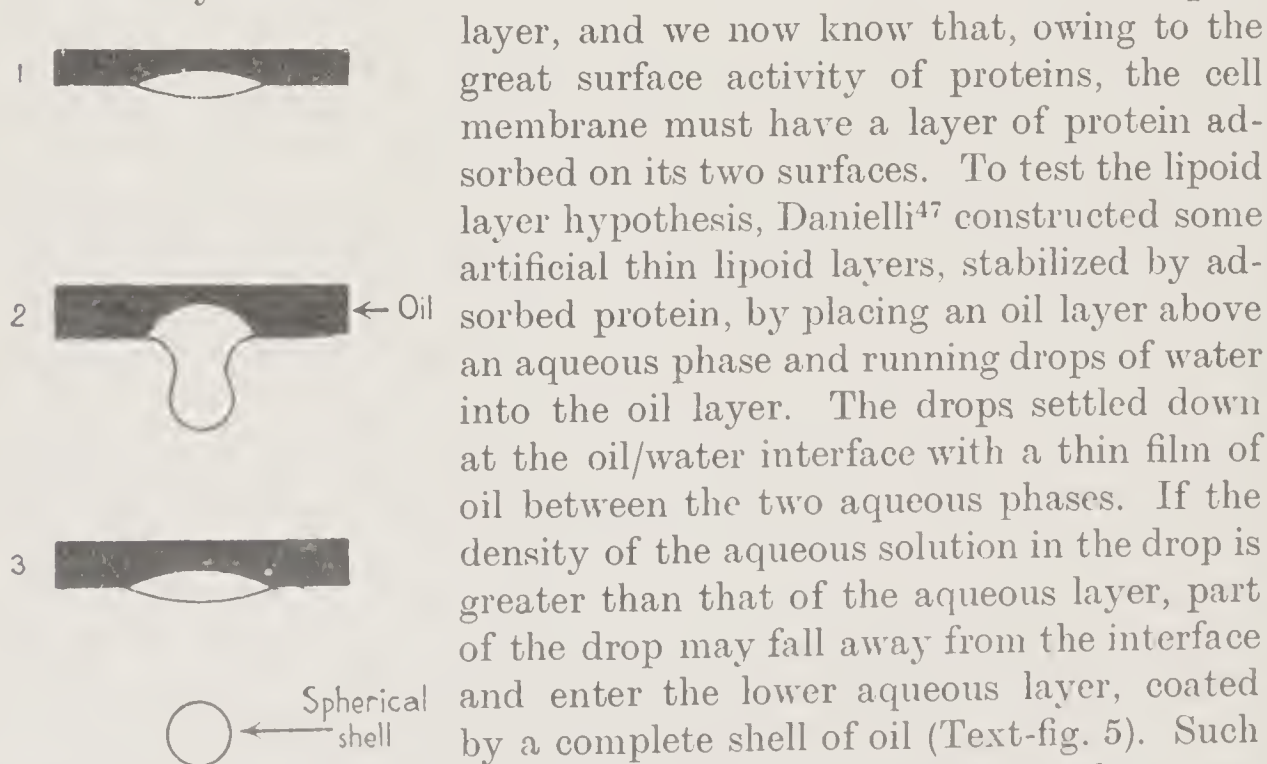
From these observations it follows that there must be at least two regions, the crystal and the gel, in which the forces operating between adjacent protein molecules may give rise to a stable or meta-stable configuration in which the protein molecules have a definite orientation with respect to one another: the intermediate positions are of relative

instability. Langmuir¹¹⁴ and Levene¹¹⁸ have discussed theoretical aspects of the problem of long-range forces between colloidal particles, which may lead to the potential energy of a particle being less at large distances of separation and over a small range δ of separation distances, than over an intermediate range of distances. The separation distance δ , though relatively small, is much greater than the distance between particles in the crystal. Thus a solution of composition corresponding to l (Text-fig. 4) should separate spontaneously into two phases, one more concentrated, the other less concentrated. The more-concentrated phase, corresponding to separation distance δ , corresponds to the spontaneously doubly refracting virus gel. Rothen¹⁵⁸ has recently produced evidence for the operation of specific forces between antibodies and antigens. The further study of these long-range forces is of the greatest importance for cytology.

These theoretical and experimental studies lay the foundations for the investigation of the rigid gel-like spontaneously doubly refracting bodies which are known to play such a large part in protoplasmic movement. Investigation of the influence of particle size, charge and hydration on these gels may throw much light on the mechanism of movement.

b. Films and Monolayers

Since Overton published his theory of the structure of the cell membrane many workers have believed that the cell membrane is a lipid



TEXT-FIG. 5. Formation of a spherical shell of oil.

layer, and we now know that, owing to the great surface activity of proteins, the cell membrane must have a layer of protein adsorbed on its two surfaces. To test the lipid layer hypothesis, Danielli⁴⁷ constructed some artificial thin lipid layers, stabilized by adsorbed protein, by placing an oil layer above an aqueous phase and running drops of water into the oil layer. The drops settled down at the oil/water interface with a thin film of oil between the two aqueous phases. If the density of the aqueous solution in the drop is greater than that of the aqueous layer, part of the drop may fall away from the interface and enter the lower aqueous layer, coated by a complete shell of oil (Text-fig. 5). Such spherical shells are relatively stable, so we can say with confidence that the lipo-protein pauci-molecular layer theory of the cell membrane is quite feasible.

Physical studies on such films, especially those not exceeding $5\text{ m}\mu$ in thickness, may reveal many facts important for biology. They may

differ from thicker oil layers in so far that in a layer only 5 m μ thick a large proportion of the membrane molecules will be oriented at the two membrane/water interfaces, whereas with thicker layers this will not be the case. At present not all the experimental difficulties have been surmounted, and the greater part of the information available on films comes from the study of monolayers at the air/water and oil/water interfaces.

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CHAPTER III

THE MONOLAYER TECHNIQUE

By J. H. SCHULMAN

i. INTRODUCTION

THIS technique is a simple and convenient way of studying:

- (1) The structure and chemical composition of interfaces.
- (2) Chemical reactions and molecular associations taking place at interfaces. The interfaces which have special biological interest are those between a monolayer and its substrate as envisaged by the air/water interface and the lipid or oil/water interface.
- (3) From a knowledge of the chemistry and behaviour of molecules of biological importance such as lipoids and proteins at oil/water interfaces direct analogies have been drawn as to their possible function *in vivo*. This has also been especially applied for those substances which have marked biological effects in minute concentrations and show a puzzling specificity. It has been shown that an analogous specificity exists between the action of these highly active substances on a synthetic cell surface as understood by mixed lipo-protein monolayers and their action on cells such as red cells, unicellular animals, or bacteria, &c.

ii. METHODS

Literature: An excellent detailed survey of all this field has been given by J. S. Mitchel²⁶ in the colloidal properties of the cell. Details of the methods used in the monolayer technique with full literature references are given by J. H. Schulman²⁹, N. K. Adam¹, and A. E. Alexander and P. Johnson³.

iii. PROPERTIES OF MONOLAYERS

a. Electrical Properties

The deposition of a monolayer of molecules at the air/water or oil/water interface changes the surface potential. This potential is that measured between an air electrode and the surface of the aqueous solution and, as will be shown later, is related in some way to the vertical component of the resultant dipole moment of the polar groups of the molecules forming the monolayer.

The most surprising and useful fact regarding this potential change is that it is constant with time. All potentials of this description should be polarized by the ions of the opposite sign in the aqueous solution or

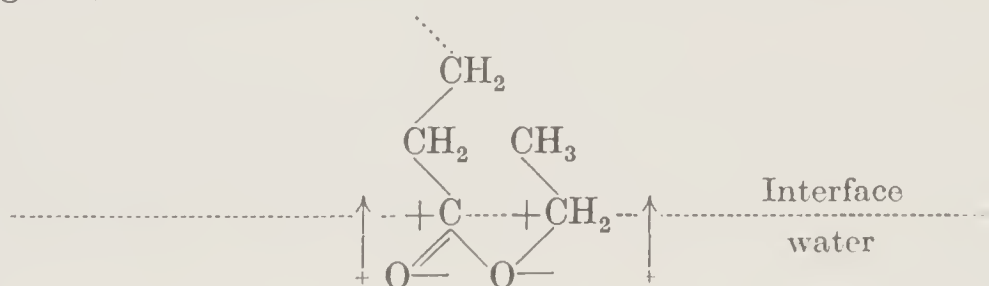
should decay with time according to the capacity and resistance of the system.

O. Gatty²⁹ suggested the following explanation for this phenomenon: the electrical double layer composed of sheets of positive and negative charges would be partially neutralized from the aqueous side of the electric double layer by ions in the solution. No polarization can take place from the air side, since any ions in the air which would become attracted to the surface to build up the neutralizing layer would be sucked continually into the solution by their own mirror image forces.

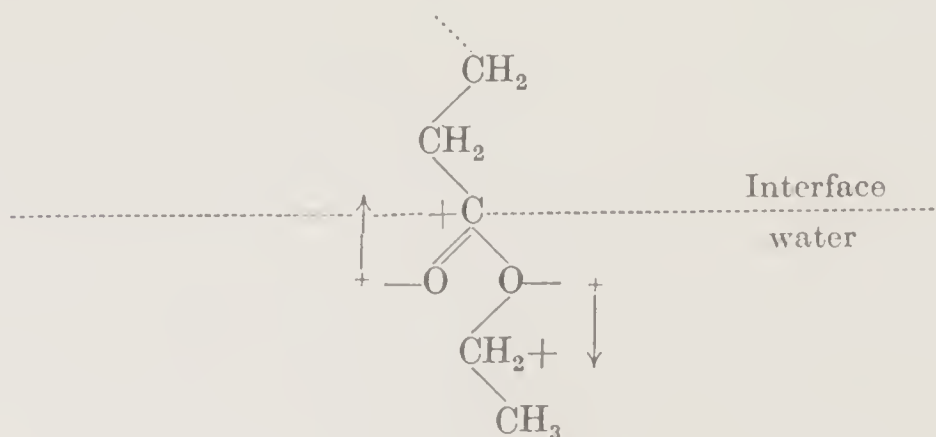
Indeed, it can be demonstrated by a suitable choice of a non-aqueous medium to replace the air, in which ions can be dissolved, that potentials of this description at an aqueous interface do decay with time according to the usual theory.

Strong indication that this surface potential is related to the resultant vertical component of the various polar groups in the molecule of the film-forming substance can be shown by the case of the aliphatic esters. Films composed of esters consisting of a long hydrocarbon chain attached to the acid radical and a short hydrocarbon chain attached to the alcohol radical such as ethyl myristate orientate themselves when the film is expanded with the two chains side by side. On compression of this film the short chain is reorientated into a position opposite the long hydrocarbon chain, thus changing the resultant electrical field of the alcohol radicle dipole⁴.

In the initial position the resultant dipole is large, as can be seen from the diagram,



and is experimentally found to be $5 \cdot 10^{-19}$ E.S.U.; in the compressed position the molecule orientates similar to the second diagram



and the resultant dipole moment as measured by surface potentials is very small, and actually in the case of cetyl palmitate is zero.

There are many other examples showing that the surface potential is related in some direct way to the polar group of the film-forming molecule. The ionization of a fatty acid film at $20 \text{ \AA}^2$ per molecule as the hydrogen-ion concentration changes in the underlying solution gives radical changes in the surface potential, starting with more than $+600 \text{ mV}$ in strong acid solutions to -150 mV in alkaline solutions³⁵. The surface-potential technique, therefore, becomes a very useful and simple tool for investigating chemical reactions and associations which a monolayer may undergo with ions or chemical substances present or injected into the underlying solution.

Examples of these reactions will be given later together with their biological analogies. Any change in the polar group of the molecule either by reorientation, association, or in its chemistry is immediately reflected in the surface potential.

The surface-potential change is given as $\Delta V = 4\pi n\mu$, where n is the number of molecules per sq. cm. at the interface and μ is a function of the polar group or groups of the film-forming molecule³⁵.

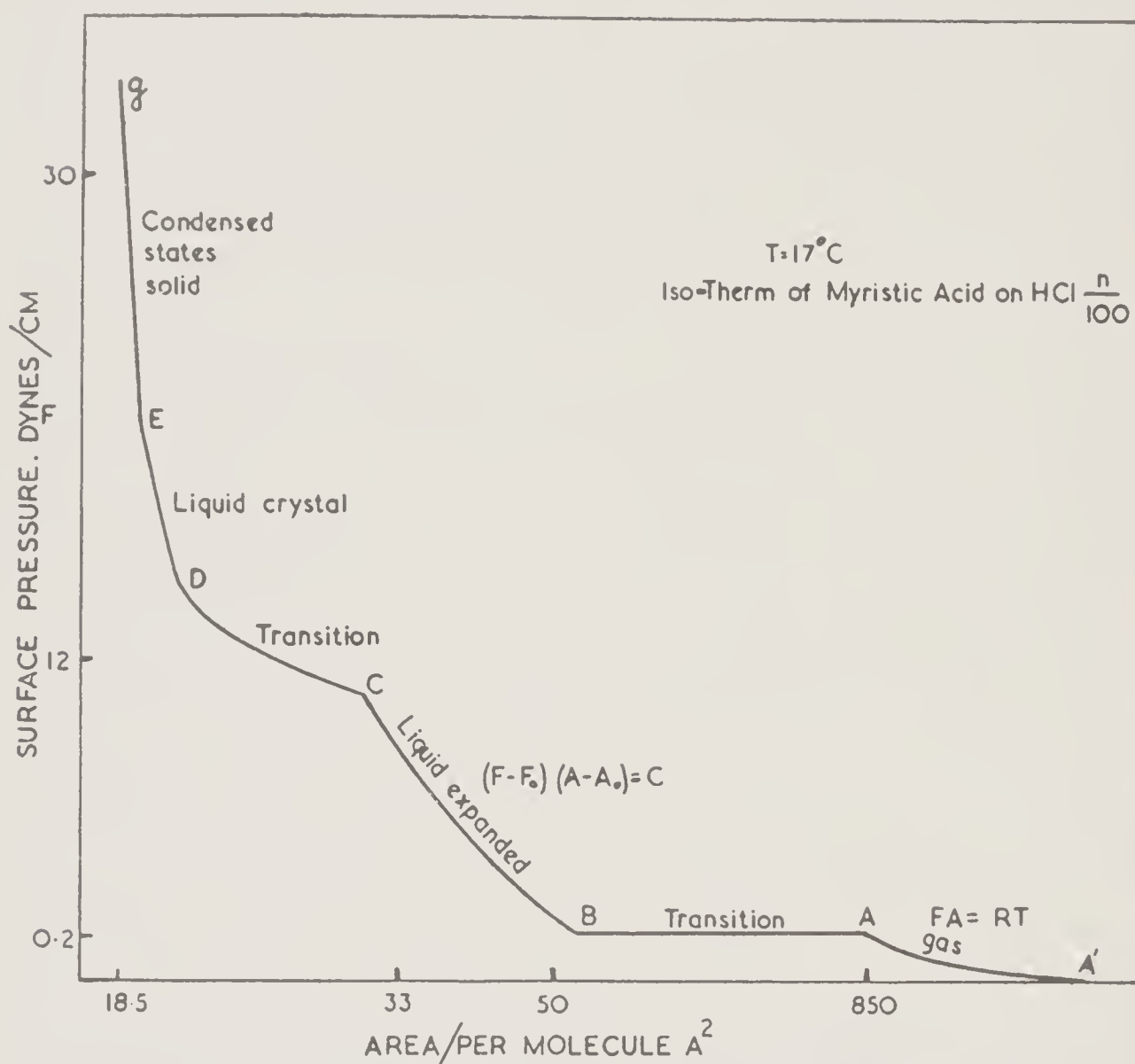
The surface potential is measured by means of an air electrode in the shape of a thin wire on which is deposited some radium D or polonium, or a millicurie of mesothorium in a tube placed in the near vicinity of the wire. These substances ionize the air gap of a few millimetres between the end of the wire and the aqueous surface. The air electrode is connected through an earthing switch to an electrometer or to a valve amplifier with a galvanometer attached. The electrometers or galvanometers are used conveniently as null instruments. This side of the circuit must be well insulated and shielded against electrical disturbances. The aqueous solution is connected to earth by means of a calomel half cell with salt bridge, or simply by means of a platinum or silver wire. Between the half cell and earth a potentiometer is arranged so that an opposite potential can be placed across the air gap to bring the electrometer needle to zero. The change of this potential due to the deposition of a monolayer is termed the surface potential of the film. The number of molecules per sq. cm. being known, μ can be plotted in electrostatic units against the area of the molecule.

The air electrode can be so manipulated as to move over the whole surface of the interface, thus establishing the area per molecule in which electrical homogeneity occurs. These areas usually denote changes of state of the film.

b. Surface Pressures

If the monolayer consisting of water-insoluble molecules be deposited on to the surface of an aqueous solution contained in a trough filled to

a bevelled edge, and the area of the film is restricted between a waxed slide and a movable waxed boom which can be kept in its original position by means of a torsion wire, then the surface pressure is defined as the force per cm. of the boom which is required to hold the boom in



TEXT-FIG. 1. Schematic isotherm out of a two-dimensional phase diagram for fatty acid monolayers on N/100 HCl.

position. Leakage of the film past the boom is prevented by means of vaselined silk threads or thin platinum ribbons.

This surface pressure (F) is plotted against the area per molecule (A) or the number of molecules per sq. cm. (N). (Text-fig. 1.)

If the surface tension of a free-water surface is γ_0 and the surface tension of the surface after deposition of a monolayer is γ_m , then the surface pressure is equal to $\gamma_0 - \gamma_m$.

This form of trough with surface boom is usually called a Langmuir-Adam trough, after the names of the two chief workers in this field. It is useful to look upon the physical characteristics of a monolayer as measured by the Langmuir-Adam trough in their three-dimensional analogies.

Thus since the insoluble molecules are restricted to one side of a barrier and their pressure measured against the free-water surface, it has all the features of a two-dimensional osmotic pressure. The barrier being semi-permeable restricts the monolayer molecules and permits the diffusion of water molecules. It is convenient to look upon the effect as due to the balance between association and ejection of the water molecules from around the polar groups of the molecules, as the monolayer is restricted between the boom and the waxed slide. If the adhesion between the molecules in the film is small, under the usual physical conditions (T , F , A), then they will obey the gas law and one may write an equation of state similar to the three-dimensional gas law.

It is actually the mutual associating forces between the polar and non-polar portions of large molecules which are the interesting characteristics of the molecules at interfaces, since it is only due to adlineation of the molecules by the asymmetrical field of forces at an interface which brings these forces into action.

In bulk solutions these forces can only be determined by viscosity and micelle formation. The energy of association between molecules when they are adlineated at an interface can be of the order of 30,000 cal., which is more than sufficient to change the chemical and, more important, the biological activity of the compound. The importance of orientation in relation to activity of the molecules will be stressed later.

As soon as the energy of the associating forces between the molecules commences to predominate over their kinetic energy, the monolayer will change its state. In fact, the force/area curve with temperature change gives a complete phase diagram for a monolayer. (Text-fig. 1.)

The various kinks in the curves are transformation points for the various states. These points have been given direct three-dimensional analogies; thus the area per molecule for the commencement of the liquid state is equal to the (volume)^{2/3} for a molecule in the interior of a drop of the liquid. The area per molecule at the end of the liquid state is the area per molecule which exists at the surface of a drop of the liquid; or the monolayer at its greatest compression in the liquid state is equivalent to a very thin drop of one molecule in thickness¹².

Complete adlineation of the molecule is required before the molecule can exist in the solid state in a monolayer.

The sharp changes and limiting areas in the force/area curves are related, therefore, to the chemistry of the non-polar portion and to the nature of the polar group, and it is reasonably easy to distinguish from the nature of the curves the possible structure of the film-forming molecule (see example later).

It is experimentally possible to measure in a simple manner changes in surface pressure from 60 dynes/cm. to 0.001 dynes/cm.; this is equivalent in three dimensions to measuring the physical characteristics

of a compound from about 100 atmospheres to one-thousandth of an atmosphere.

Two-dimensional Gas State. It can be seen in Text-fig. 1 that at very low pressures for the example given (Myristic acid below 0.2 dynes/cm.) there is an isotherm AA' obeying the law $FA = RT$, which is equivalent in three dimensions to $PV = RT$. Equally one may write an analogue to the equation of Amagat $F(A - A_0) = XRT$, where A_0 is a co-surface area of the molecular similar to the three-dimensional co-volume, and X is a constant. This permits one to measure the molecular weight of the molecule by simply plotting the C/F (C surface concentration) ($1/A = N$ number of molecules per sq. cm.) against the surface concentration. (X relates to the non-ideality of the gas.) Then

$$M = \text{molecular weight} = 2.43 \times 10^6 \lim_{c \rightarrow 0} C/F.$$

On compression of this two-dimensional gas liquification will take place at a certain pressure, temperature, and area. (Myristic acid 17° C., 0.2 dynes/cm. and 850 Å.) An equilibrium pressure will set in which, on restriction of the area of the surface film, forms a transition from a gas to a liquid phase. Thus transition consists of a two-dimensional liquid micelle in equilibrium with diffuse gas molecules. The flat transition at constant pressure (AB) can at certain temperatures deviate from a flat horizontal curve if the liquid micelles are sufficiently small to exert a type of gas pressure on their own^{15, 16}.

At an area of approximately 50 Å² for myristic acid at point B (Text-fig. 1) the whole surface is now uniformly covered by the two-dimensional liquid, as shown by a sudden rise in surface pressure and surface potential. This potential is now uniform over the whole surface, in comparison with the range AB , where the surface potential can vary from 150 mV to 10 mV, as ascertained by the moving ionizing air electrode³⁵.

Two-dimensional Liquid State. The compression range of the monolayer from 50 to 33 Å² (BC , Text-fig. 1), although comparable to a liquid, varies from a true liquid in that it consists of a layer of orientated hydrocarbon chains adhering together through Van der Waals forces, together with a layer of carboxyl polar groups associating with the water, but repelling one another. Thus the duplex film behaves as a liquid in the hydrocarbon layer, and a gas in the polar layer. An equation of state²⁴ has been given for this type of force/area curve:

$$(F - F_0)(A - A_0) = C.$$

F_0 is related to the lateral adhesion between the hydrocarbon chains on the non-polar portion of the molecule, and A_0 is related to the cross-sectional area of the polar group.

Considerations have also been given in that on compressing a mono-

layer, besides changing the tilt of the molecule to the horizontal, one is restricting the rotation the molecules may undergo around their long axis.²² At point *C* 33 Å² (myristic acid T. 17° C.) a transition from the liquid to the solid or condensed state takes place. This transition is similar to a liquid in equilibrium with a solid, and should be a flat constant-pressure transition. The fact that the curve *CD* is not horizontal is explained again in that the liquid micelles are sufficiently small to exert a pressure on their own. Point *C* could also be a higher transformation point, e.g. a configuration or reorientation change in the molecule without change of energy. In this case, restriction of rotation of the molecules occurs along the main axis. In most cases the range *CD* is electrically unhomogeneous by surface potentials, favouring the first theory.

Two-dimensional Condensed or Solid States. The curve *DEG* can be seen from its slope to denote a highly incompressible monolayer with the limiting area of *ca.* 19 Å². This area is that of the cross-section of a vertically orientated hydrocarbon chain as established by X-ray diffraction patterns for fatty acid crystals. The portion *EG* of the curve is usually a two-dimensional solid, as shown by the fact that talc powder particles do not move when blown upon on the surface, or only in short jerk-like movements. The portion *ED* can represent a two-dimensional liquid crystal in which the molecules have only one common orientation. The association of water molecules around the polar group, and the mutual association of the polar groups themselves, can obviously play an important part in the structure of the condensed films. If there is strong mutual polar-group association the water molecules can be squeezed out from between the head groups. This is especially marked with the long-chain alcohols, where hydroxyl bonding can interlink the polar *OH* groups, and it has the somewhat astonishing effect of restricting the evaporation of water from the underlying solution. A compressed film of octadecyl alcohol can restrict water evaporation from the surface to within 2 per cent. of that of the free-water surface⁴². It can be seen from Text-fig. 1 that, on lowering the temperature, an isotherm can be obtained in which a triple point exists between solid, liquid, and gaseous phases. This temperature would denote a two-dimensional melting-point, which when compared to the three-dimensional melting-point, usually coincides with a vitreous melting-point. With the alcohols the two-dimensional and three-dimensional melting-points coincide¹².

c. Surface Viscosity

Owing to the high degree of orientation of the molecules at an interface very high surface viscosities are obtained for surface films. A knowledge of the change in surface viscosity with area per molecule gives useful information on the structure and associating properties of the

compounds which form monolayers. This is especially important for protein films. Two standard methods are used which give results for viscosity changes, but information on the absolute viscosity is difficult to obtain, and the theory is still being developed.

One method is to measure the time necessary to flow a monolayer through a narrow canal under a weak pressure gradient. A further method is to oscillate a vane in the surface and measure the logarithmic decrement of the successive oscillation produced by the clean surface and the surface covered by the film.

For very narrow canals the analogy with a two-dimensional Poiseuille's Law holds; where the quantity of film Q passing in unit time $Q = Fd^3/2\eta l$ where η = surface viscosity, d = width, and l = length of canal. If the surface viscosity η is plotted against the area per molecule of the film-forming substance, the kinks obtained in the curves are usually at the same area as shown by the surface potential area curves. The kinks are apparently at higher order transformation points, and denote reorientation effects in the molecule on area change^{21,22}.

d. Multilayer Formation

Information regarding the area and associating properties of molecules can be obtained from the three described techniques, but it is difficult to determine the thickness of the layer or the vertical dimension of the molecule forming the monolayer. The multilayer technique is a simple way of determining this factor. If a slide which has a hydrophobic surface or is made hydrophobic by the deposition of the first monolayer is pushed through the surface of the aqueous solution upon which a monolayer is kept at constant surface pressure, independent of the area of the monolayer, then under certain conditions the monolayer will be deposited on to the slide.

The physical condition necessary for deposition of the monolayer is primarily that of the contact angle which the covered aqueous solution makes with the penetrating slide. This contact angle can be readily controlled by changing the surface pressure of the film and also by varying the chemistry of the polar group of the film-forming molecule⁷.

The film can only be deposited on the slide if the contact angle is obtuse; in general at low surface pressures this angle is obtuse only on the downward movement of the slide through the surface of the aqueous solution and not on the upward movement. Hence deposition in this case will occur on the downward movement and is termed *X* deposition. At high-surface pressures the contact angle is obtuse both on the downward and upward movement of the slide through the surface, the film being deposited on both movements. This deposition is termed *Y* deposition. On repeated movement of the slide through the surface the films will be rolled on to the surface of the slide according to the above

conditions. The surface pressure of the film on the aqueous solution is conveniently kept constant by so-called piston oils. Lenses of these oils such as oleic acid (30 dynes/cm.), castor oil (16 dynes/cm.), tritolyol phosphate (10 dynes/cm.), keep a constant surface pressure with the film of these substances irrespective of the area of the film so long as there is excess of the oil on the surface in the nature of a lens. The depositing film and the pressure film are kept separated by a vaselined silk thread.

The molecules in the consecutive layers will orientate themselves irrespective of the method of deposition into double-layer or single-layer lattices according to their crystal structure.

The built-up multilayers are half-way between an amorphous mass and full crystallization, the molecules in the consecutive layers having only one common axis, that normal to the surface. This orientation holds throughout the whole thickness of the multilayer, even up to a thousand layers²³. As soon as the thickness of the multilayer is equal to a quarter wave-length of light, Newtonian colours set in when they are seen in polarized light at a low angle of incidence. This is a convenient way of measuring their thickness; the colours when viewed in white light, or their intensity when viewed in monochromatic light, are sensitive to changes of only one molecule in thickness.

Since the polar groups in molecules give clusters of atoms heavier than the CH_2 group at definite spaced intervals, an X-ray beam will be reflected from these planes as in a Bragg diagram obtained from an ordinary crystal. These main spacings obtained by permitting a monochromatic X-ray beam to travel along the multilayer at small angles of incidence give the thickness of the layer in the multilayer and the tilt of the molecule. Under careful X-ray examination of a thick multilayer the side spacings between the molecules can also be determined.

Substances which do not crystallize in bulk but only form greases can be made into two-dimensional crystals as in monolayers. These monolayers after being built into multilayers will give an X-ray diagram showing all the atomic spacings in the molecule. An X-ray diagram of a multilayer taken at one angle of incidence gives a pattern identical with a rotation picture of the crystal of the substance.

The value of this technique in determining the structure of a non-crystallizing substance obtainable only in minute quantities in combination with the other three surface techniques is considerable.

iv. THE STRUCTURE OF PROTEIN AND LIPO-PROTEIN FILMS

a. Protein Films

It is an astonishing property of proteins in general that solutions or solid particles of them spread at the air/water or oil/water interface to form a very thin film. This film is insoluble, except under certain

conditions, in the underlying solution, although the protein itself is very soluble in the underlying solution.

Furthermore, these films upon compression develop very high viscosities and ultimately become so-called two-dimensional gels of varying rigidity and elasticity.

The suggested explanation for the formation of films of this type is that the globular protein molecule in solution unfolds at the interface owing to the asymmetrical forces. This permits the non-polar side chains to orientate and associate together away from the side chains with polar groups, leaving the backbone composed of the —CHR—CO—NH— chain separating them.

The picture created at the interface is thus of a triplex layer with non-polar chains in the air or oil phase, the backbone chain with the polar side chain drawn into the aqueous phase. Association by short-range forces between these three groups either intra- or inter-molecularly is probably responsible for the large molecule remaining at the interface and not dissolving away into the underlying solution.

Adlineation between the large molecules lying on the surface at high surface compression is probably responsible for the formation of the gels and films of very high viscosity. As can be expected, the rate of spreading of proteins and the properties of the film are dependent on the hydrogen-ion and salt concentration of the underlying solution and on the chemical nature of the proteins. But in general a film of a protein becomes coherent by surface-potential measurements and exerts a surface pressure of 0.2 dyne/cm. at a weight of 1 mg. per sq. metre.

At this area the films are fluid and on compression they gel and start showing strains in the film in the form of striations at about a third of this area.

This force/area curve is reversible except when the film is held for any length of time at high pressures. High surface pressures force the film molecules into solution again. Whether only the smaller molecules in the film as breakdown products of the large protein molecule or whether the whole film can be redissolved by these surface pressures is still a matter for research.

A highly compressed film showing striations is completely irreversible. The striations are micro-fibres, and they can actually be spun off the surface of the water into insoluble and non-spreading threads⁹.

Proteins from the solid spread least of all at their iso-electric point, but a film spread at another pH is quite stable at its iso-electric.

Proteins from solution only spread better at their iso-electric in an artificial manner, since in comparison with solutions of other pHs they are least soluble and the protein appears at the surface from diffusion and solubility considerations quicker in an iso-electric solution than in a solution in which they are readily soluble. It is possible to overcome

all spreading difficulties by dissolving the protein in suitable salts in the presence of iso-propyl alcohol. The salt and the alcohol spread the protein in a manner similar to the spreading of stearic acid with benzene, &c. The association between the molecules is broken down by dispersion and the alcohol artificially spreads the molecules by reason of its own spreading forces.

These solutions of proteins spread to films independent of the nature of the underlying solution. The protein molecule at the interface is distorted by reason of the separation of the non-polar side chains from the polar side chains. The continuation of the fundamental unit of the backbone CHR—CO—NH— is further complicated by the presence of proline derivatives. These factors would necessitate the protein backbone to form loops instead of a continuous zigzag or any of the theoretical pictures of the α and β keratin type.

On compression of the film these loops would be forced out of the surface and are possibly responsible for the formation of the striations in the film in the form of hydrophobic folds. They are also responsible for the fact that the repeat unit CHR—CO—NH can be compressed to $10 \text{ \AA}^2$ in the film, which is well below the theoretical area possible of $15.7 \text{ \AA}^2$.

Adlineation or disorder of the long molecules extended on the surface at low concentration or surface pressures possibly gives rise to the different types of force area curves found by many workers in this field. Indeed, there is evidence from low-pressure and surface-viscosity work for these two types of films, according to whether time is permitted for adlineation to take place. It is possible by measuring very low surface-pressure area curves to determine the molecular weight of the protein molecules. This is done in a similar manner to the three-dimensional osmotic pressure, by plotting the c/p against the surface concentration ($p =$ surface pressure) in highly dilute systems. The results are astonishing in that they show that the surface molecular weights are similar to the bulk molecular weights, in spite of the surface denaturation^{15, 16, 17}. The formation of gel films of a reproducible nature seems to be the characteristic of substances of high molecular weight at an interface, since the esters of polysaccharides or synthetic resins form films very similar to the proteins.

An interesting difference is that the threads spun off the surface of the compressed monolayer of a polymer like polyvinylacetate are very elastic, and can be drawn out considerably in comparison with the rather brittle protein threads. This possibly denotes an inter-association of the polar groups in the protein, which is absent in the case of the polymers.

b. Lipo-protein Films

In view of the fact that certain proteins such as gliadin are soluble in 60 per cent. alcohol, and that most proteins can be dispersed in salts

and iso-propyl alcohol solutions, lipoids can be added to the protein solutions. Mixed lipo-protein films can therefore be prepared with varying proportions of lipid to protein.

Cholesterol and gliadin mixed films have been made in this manner, and they show very interesting properties in relation to their surface viscosity. A gliadin film on a buffer solution of pH 7.4 becomes coherent at about 1 mg./m.² and commences to gel strongly at about 16 dynes/cm. On further compression the gel becomes more rigid until the irreversible film with striations appears.

Incorporation of 20 per cent. cholesterol to the gliadin has a striking effect on the force area curve. At 16 dynes/cm. the mixed film gels as before, the gelation becoming more rigid on compression until at about 21 dynes/cm. the rigid film suddenly liquefies. This liquefaction on compression and solidification of the film on expansion is a reversible process. The compressed liquefied film has all the properties and characteristics of a cholesterol film.

It could thus be supposed that at 21 dynes the cholesterol molecules in the monolayer of protein push the protein molecules into the underlying solution in the form of a double layer. On release of the pressure the associating short-range forces between the polar group and non-polar portion of the cholesterol molecule and the similar groups of the protein molecule permit the protein molecule to penetrate into the cholesterol upper film and thus re-establish the mixed monolayer. Other examples of penetration and adsorption of molecules into and on to monolayers will be given in the next section.

It has been shown that this sudden liquefaction phenomenon on compression of a mixed lipo-protein film is related to the salt concentration and pH of the underlying solution and also to the lipid protein ratio. It was found that the greatest change in surface viscosity is obtained with a 20 per cent. lipid concentration, which is similar to the lipid concentration occurring in natural membranes³⁸.

V. INTERACTIONS OF MONOLAYERS WITH SUBSTANCES INJECTED INTO THE UNDERLYING SOLUTION, AND THEIR BIOLOGICAL ANALOGIES³⁹

a. Penetration of Lipoid and Protein Monolayers

Research in this field consisted mainly in forming monolayers at interfaces composed of substances which exist in natural membranes, and injecting into the underlying solution substances which are known to react with these membranes. A proof of the analogy with biological systems was achieved by an identity of the specificity of the synthetic and biological reactions.

If certain soluble capillary active substances are injected in minute concentrations (10^{-4} per cent.) under a monolayer kept at a constant

area and there is association by short-range forces between the film-forming molecules and the injected molecules, great changes in surface pressure, surface potential, and viscosity of the monolayer are observed. Furthermore, if the monolayer is kept at a constant pressure, great changes in the area of the monolayer are observed to take place.

These changes are shown to take place when the injected substance does not in itself affect the free surface of the aqueous solution to any great extent.

It has been shown that this reaction is very specific, and is due primarily to an interaction between the polar groups of the film-forming molecule and the injected molecule and, further, to association between the non-polar portions of the two molecules.

The polar group interaction anchors a molecule from the solution, thus permitting the non-polar portion to penetrate into the monolayer if there is association between the non-polar portions of the molecules. On penetration of a monolayer, therefore, the number of molecules is increased, and since the stability of the complex is greater than that of either of the two components alone, an equimolecular mixed film will result, which will stand greater surface pressures than either single component.

This explains why on penetration the surface pressure rises to very high values, since the number of molecules in the monolayer has been doubled and the area kept constant. Further, the surface potential will rise or fall according to the magnitude of the penetrating polar group and its association with the polar group of the film-forming molecule.

b. Polar Group Specificity

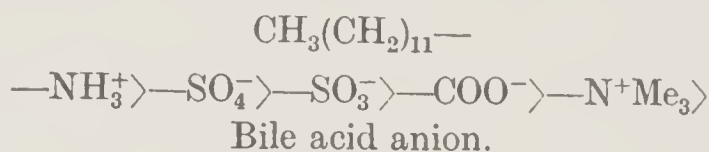
Excellent examples showing the specificity in reactions involving primarily the polar groups are given by the penetration of cholesterol and cholesterol-ester monolayers by long-chain hydrocarbon ions^{38, 39}.

If a cholesterol monolayer be compressed to 2 dynes/cm. and a minute quantity of sodium hexadecyl sulphate be injected into the underlying solution, the surface pressure of the cholesterol monolayer rises within 2 minutes to *ca.* 55 dynes/cm.; but if a film of cholesterol stearate or acetate be taken instead, the surface pressure rises only slightly above that of the sodium hexadecyl sulphate solution alone in the same time.

This penetration of the cholesterol monolayer was attributed to a complex resulting between cholesterol and sodium hexadecyl sulphate, since a solution of saponin under identical conditions also raises the surface pressure of the cholesterol film to 60 dynes/cm. and solidifies the film. This reaction is completely stopped with a film of cholesterol acetate. The saponin complex with cholesterol is well known, and it is interesting that saponin has been shown not to penetrate any other film such as lecithin, long-chain alcohols, calciferol long-chain phenols,

sphingomyelin, &c., with which it is known not to make complexes. It is further shown that if equimolecular solutions of long-chain paraffin ionic compounds of identical chain length be injected into a solution under a cholesterol film held at a constant area, then a reactivity series could be established with the different polar groups as measured by the equilibrium surface pressure of the cholesterol film.

This series is the following^{32, 39}:



The long-chain C_{12} amine salt penetrates a cholesterol film to the extent of 50 dynes/cm., and the bile acid salt has very little effect on the cholesterol film at all. The significance of these reactions will be demonstrated in the examples quoted later for their cytolytic action.

It appears that the attractive and repulsive forces between polar groups may be interpreted upon the hypothesis that they are due to coulomb forces acting between polar groups in systems containing ions and dipoles, an $\text{ion}^+ \text{- ion}^-$ having a greater energy of association than an ion-dipole , which again is greater than a dipole-dipole , and repulsion taking place between ions of like sign. It is these forces which are responsible, together with the non-polar associations, for the physical state of mixed films. Mixed films can be partially ionized acids or bases or two component systems such as acids and bases or mixtures of these with alcohols, ethers, esters, &c.

c. Non-polar Specificity

The energy of the non-polar association depends on the number of CH_2 groups or equivalent groups one can orientate and adlineate between molecules in a monolayer. Thus, if 16 (CH_2) groups can be orientated opposite one another in molecules in a monolayer as in a crystal, the energy of association is equivalent to strong polar group interaction. The energy of association is dependent on the sixth power of the distance separating the carbon atoms, hence change in the adlineation due to stereochemical configurations would have a very great effect on the association between the molecules. These factors are well demonstrated in practice.

The penetration of a monolayer of cholesterol or long-chain paraffin alcohol of sixteen carbon atoms by the salt of a long-chain sulphate is very different, according to the number of carbons in the penetrating molecule. Thus it only requires a surface pressure of 8 dynes/cm. to eject C_{16} long-chain sulphate molecules from a mixed film containing cholesterol, but already 35 dynes/cm. is required to eject C_{18} long-chain sulphate molecules. Similarly, great differences are observed between

long-chain compounds containing a double bond according to whether the arrangement is in the *cis* or *trans* configuration⁴¹. The molecules of the *cis* compounds cannot adlineate either with themselves or other molecules in the *trans* configuration. Therefore on compression of a mixed film of a *cis* and *trans* compound ejection of the molecules of the *cis* compound will take place if the polar association between the molecules is weak.

This is again reflected in penetration experiments. Only very poor penetration of a monolayer of molecules in the *trans* configuration by molecules in the *cis* configuration can take place, and likewise for the inverse order.

No ejection of a component takes place in a mixed film where strong association exists. Such films collapse as a 1 : 1 unit at very high surface pressures. It is possible to apply a two-dimensional phase rule to mixed and penetrated monolayers.

d. Penetration of Protein Films

Long-chain paraffin ionic salts of the description mentioned in this section have the property when injected into solutions under protein films of liquefying the protein film and ultimately dispersing it.

This occurs in very small concentrations; and the reactivity is similar but not so marked as the series quoted for penetration into cholesterol films.

The explanation given for this phenomenon is that the large protein molecules are associating in the form of a network through polar groups of the —NH_3^+ and COO^- type and also by their non-polar side chains. These associations will be broken down by competition from the polar groups of the penetration molecules. These smaller units will be deterged or displaced from the surface by the injected solution of the long-chain paraffin salt, which usually lowers the interfacial tension to a greater extent than the protein film. The bile acid salts seem to have no effect on protein films as with the sterol films, only associating with fatty acids.

*Penetration of Lipoid Monolayers by Proteins*³³. An interesting experiment is shown by the injection of very dilute protein solutions, 1 mg./150 c. under certain lipoid films, in which association between the lipoid and the protein molecules can be established. As with the other penetration experiments, very high surface pressures are obtained; these are much greater than the lipoid film itself can withstand. Even more astonishing is the fact that proteins which collapse readily at 16 dynes/cm. on their own, will penetrate lipoid monolayers and hold surface pressures as high as 40 dynes/cm. The penetration of cholesterol monolayers by proteins takes place over the whole range of pH and by most proteins. Lecithin monolayers, however, are not penetrated, except in pHs more acid than 3 and more alkaline than 11. Cephalin monolayers

are only penetrated with proteins on the acid side of their isoelectric point, and not at high pHs. Likewise, films of certain phosphatidic acids (such as Kahn antigen) are only penetrated by a positively charged protein molecule, and only poorly by this molecule in alkaline pHs.

Penetration of long-chain amine and substituted amine monolayers by proteins such as serum proteins, shows a puzzling specificity. It is interesting that if a solution of a pH is chosen which inhibits a penetration of a lipid monolayer by a protein, and the pH is changed to an associating media, penetration rapidly follows. Ejection of the protein molecule takes place when the pH is brought back to its original value. Likewise penetration at constant pressure permits the protein to expand the monolayer to double its original area; this protein can be partially ejected³³.

e. Adsorption on to Protein Monolayers

Penetration has been shown to change the surface pressure of the film and the surface potential. Adsorption, being primarily an association of the polar groups of the film-forming material and the injected substance, is shown only by a change in the surface potential and the viscosity or rigidity of the film.

The interest of this technique is the adsorption of material on to protein films. It is shown that the chief reacting group in the protein monolayer is the free NH_3^+ group, since most reactions undergone by protein monolayers are very similar to long-chain amine films. The carboxyl and keto imido polar groups play a secondary role in the reactivity of the protein films. One of the most interesting reactions studied is the action of compounds with phenolic groups such as tannic acid and *p-p'* dihydroxy stilbenes, &c., on to protein monolayers. The reaction is related to the number of phenolic groups and their spacing per molecule of adsorbing compound.

Thus, gallic acid adsorbs very slowly on to a protein monolayer, but tannic acid in very dilute concentration immediately reacts, changing the surface potential of a gliadin film from 360 to 200 mV. This tanned monolayer loses all its elasticity and behaves as a skin on the surface. It is also interesting that such a tanned monolayer can no longer be dispersed by long-chain paraffin ionic compounds such as sodium oleate, as explained in the previous section³⁸. But the gallic acid-treated protein film can be dispersed in the usual way. This tanning reaction is very sensitive to pH, optimum tanning taking place between pH 7-8; very little tanning takes place beyond pH 4 and 9.5⁸.

It is supposed that the multiple-point contact of a tannic acid molecule interlaces the protein film by associating with the ionized amine polar groups in the film, thus preventing dispersion of the film and giving it extra rigidity.

The rate of the reaction is dependent on the number of reacting groups

per adsorbing molecule. A long hydrocarbon chain amine film is tanned in a similar way, but with a great expansion of the amine film; this does not take place with the protein films.

Pyrogallol is not adsorbed by protein films, but its polymerized product purpuro gallin is strongly adsorbed⁸.

The *p-p'* dihydroxy stilbene and related compounds, which are synthetic oestrones, behave in an interesting manner, since with addition of side chains the adsorption increases markedly per added CH₂ group. The diethyl gives the maximum adsorption, since any further addition of CH₂ groups changes the adsorbing molecule into a protein film dispersant. It is interesting that the biological activity is at a peak with the maximum adsorbing compound. The dispersing properties of the compound coincide with the marked fall in biological activity.

f. Cytolysis and Agglutination

It was quickly seen in seeking for biological analogies between the monolayer and biological reactions that reactions taking place at cell surfaces were good examples, such as the cytolysis of red cells and unicellular animals.

All compounds examined that penetrate cholesterol monolayers or disperse protein films haemolyse, and all compounds that only adsorbed on to protein films without dispersing them agglutinate red cells.

Furthermore, the same reactivity series for long-chain hydrocarbon compounds in penetrating cholesterol monolayers is very similar to the reactivity series obtained with these compounds in their haemolytic activity, as measured by the minimum concentration necessary to produce haemolysis.

Thus, long-chain amines showed maximum haemolysis and the bile acid salts were not haemolytic at all³⁹.

Some of the unicellular animals such as *Paramecia* showed likewise a very similar behaviour to the red cells; instead of lysis, lethal activity as measured by movement of the animals was considered.

They were killed most readily by the long-chain amines and least by the bile acids, and showed distinct cytolysis when dead.

Those substances which adsorbed on to protein monolayers without dispersing them agglutinated the red cells and killed the *Paramecia* without cytolysis.

These substances are the phenolic compounds as described, gallic acid having little action, but tannic acid is active in low concentrations. Likewise, the *p-p'* dihydroxy-stilbene derivatives gave very similar values as to their adsorption on to protein films and their lethal action on *Paramecia*. The compounds as described which showed protein film-dispersion properties were haemolytic, and cytolysed the *Paramecia* in very low concentrations.

Rotifera, another microscopic animal, seemed to be immune against the substances mentioned here.

In view of these results it could be supposed that, since substances which penetrate cholesterol films and protein films are haemolytic and the substances which only adsorb on to protein films agglutinate red cells, there must be cholesterol and protein available on the red cell surface. It could thus be imagined, therefore, that the cell surface was a mixed cholesterol/protein film. A similar surface would be feasible on these grounds for the *Paramecia* coating. The *Rotifera*, which appeared to resist these substances, must have another surface, such as chitin, which would be unimpaired by the lytic or adsorbing substances described here.

g. Induced Permeability of Cells by Adsorption of Long-chain Compounds

The action of long-chain compounds in promoting the lysis, growth inhibition, and growth acceleration of bacteria and other cells has been correlated with the oil solubility and surface activity of these compounds. This correlation between surface activity and oil solubility on the one hand, and biological activity on the other, has been demonstrated for many homologous series of compounds, and as a rule it may be said that increased oil solubility and surface activity are accompanied by increased biological activity up to a certain optimal point. Beyond this optimal point correlation breaks down, and further increase in the oil solubility and surface activity within the series does not further enhance the biological action.

In general, the action of these compounds is accompanied by gross changes in the cell membrane, resulting eventually in the complete lysis of the cell. This lytic action has tended to obscure the fact that the biological activity of these compounds may not in reality be due to their surface activity at all, but rather to some more fundamental structural properties of their molecules—in other words, the correlation between surface activity and biological activity in these compounds may be completely accidental, and surface activity may be in no real sense the cause of biological activity. This possibility becomes more obvious when one considers the fact that many of these long-chain compounds, especially those with two or more terminal polar groups, have very little surface activity, and yet produce remarkable biological effects, even at concentrations of only a few γ .

It can be shown from the surface chemistry of these compounds that orientation, complex formation, association, and adsorption of the molecules—factors in no way related to their surface activity or oil solubility—all play an important part in their biological activity⁵. From the biological point of view perhaps their most important property is that of being adsorbed on to the cell surface, and changing the perme-

ability of the cell membrane, even at very low concentrations and without causing any immediate change in the *in vitro* respiration of the cell. In view of this change in permeability, the environment of the cell whose reaction to these compounds is being tested becomes of great importance, and discrepancies between *in vitro* and *in vivo* tests are only to be expected. In fact, the toxic action may be due to the long-chain compound only in so far as it induces the permeability of the cell to compounds which normally cannot gain access to the cell, or permits the leakage away from the cell of substances which cannot diffuse through the intact membrane.

Examples for yeast cells in the environment of different organic acids and their salts have been worked out at different pHs in the presence of active agents such as long-chain diamidines, and salts of methyl-substituted fatty acids. These compounds are very active in inducing the permeability of the yeast cells without effecting their respiration in dilute concentrations³⁷.

vi. THE OIL/WATER INTERFACE

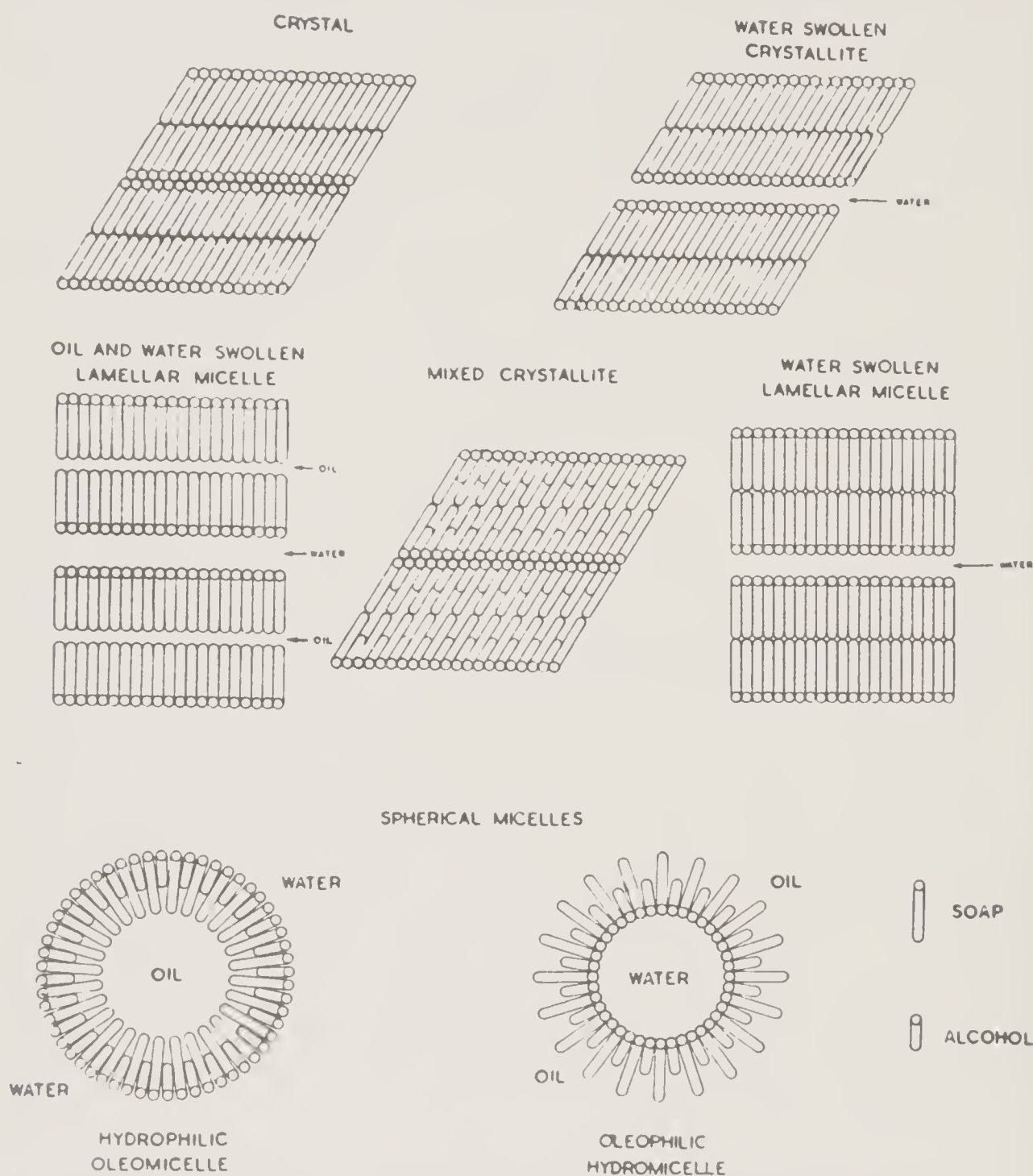
a. *Crystal, Micelle, Swollen-micelle, Emulsion*

A knowledge of the interactions and associations molecules may undergo at the air/water interface, as studied by the Langmuir trough, greatly assists in interpreting and foretelling how molecules will interact, or associate, at the oil/water interface, and aggregate at the micelle/water interface. The competing association of the oil phase molecules with the film-forming molecules brings into play a new variant as compared to the air/water interface. A simple example of the significance of these oil/water interfacial forces can be shown by the behaviour of long-chain alcohols and fatty acids at a benzene/water interface, in comparison to an air/water interface¹⁹. At an air/water interface, the two molecules can pack to an area of approximately $20 \text{ \AA}^2$, whereas at the oil/water interface the long-chain alcohol readily packs to an area of $20 \text{ \AA}^2$. The acid in the presence of benzene, &c., packs to a minimum area of $40 \text{ \AA}^2$. This shows that the association benzene/fatty acid is greater than fatty acid/fatty acid, whereas the hydroxyl bonding in the alcohol enables the long-chain alcohol molecules to pack with sufficient cohesion to resist penetration by the non-polar solvent molecules. The laws governing the packing and mutual cohesion of the molecules at oil/water interfaces, together with penetration of the non-polar or polar portion by oil or water, control the whole phenomenon of emulsions.

b. *Micelle Formation*⁴⁰

It can be shown in Text-fig. 2 how a stabilized droplet of water in oil or oil in water can be formed, starting from a crystal of soap molecules.

The inclined bimolecular layers of soap molecules in a crystal can adsorb a monolayer of water molecules on each polar force of the leaflets and still remain a crystal. If this water-swollen crystallite be heated in



TEXT-FIG. 2. The phenomena of solubilization of soap crystal lamellar by non-polar oils and penetration of the monolayer lattice by polar oils in the formation of micelles and emulsions.

water the whole opaque system at a certain temperature (Krafft point) becomes clear. It can be seen from X-ray data that at this point the molecules can rotate about their main axis and become vertically orientated, instead of inclined. This extra molecular disorder enables the polar layers in the lamellar to separate from one another on dilution of the soap with more water. This is seen by the increasing X-ray

spacing given by the scattering from the parallel-arranged polar sheets, which are moving apart. A constant X-ray spacing is also given by the dimension of the bimolecular leaflet. As the soap is therefore diluted (above the temperature of the Krafft point) the increasing X-ray spacing becomes very large, and finally disappears at a concentration about ten times greater than the micelle-point concentration. The bimolecular leaflet X-ray spacing also fades out at about the same concentration¹⁸.

Another X-ray spacing can be demonstrated in soap solutions, which sets in at concentrations somewhat greater than the micelle point. This is small and related to a side spacing between the molecules in the monolayer lattices of approximately 4.8 Å. This single-side spacing compares to the two side spacings of about 4.2 Å and 4.5 Å found in the soap crystal, showing that at the Krafft point the soap in micelle form must be in a liquid crystal state, and that the molecules in the micelle must have greater order than in a liquid, but less than in a crystal. This will be seen to be important in the formation of emulsions. It has been established by physical chemical methods, and by light-scattering methods, that the micelles in dilute soap solutions contain about 80 molecules¹¹. It is supposed that, on a concentration increase, these micelle 'boxes' pack together, producing a streaming birefringent system giving the above X-ray diffraction spacings.

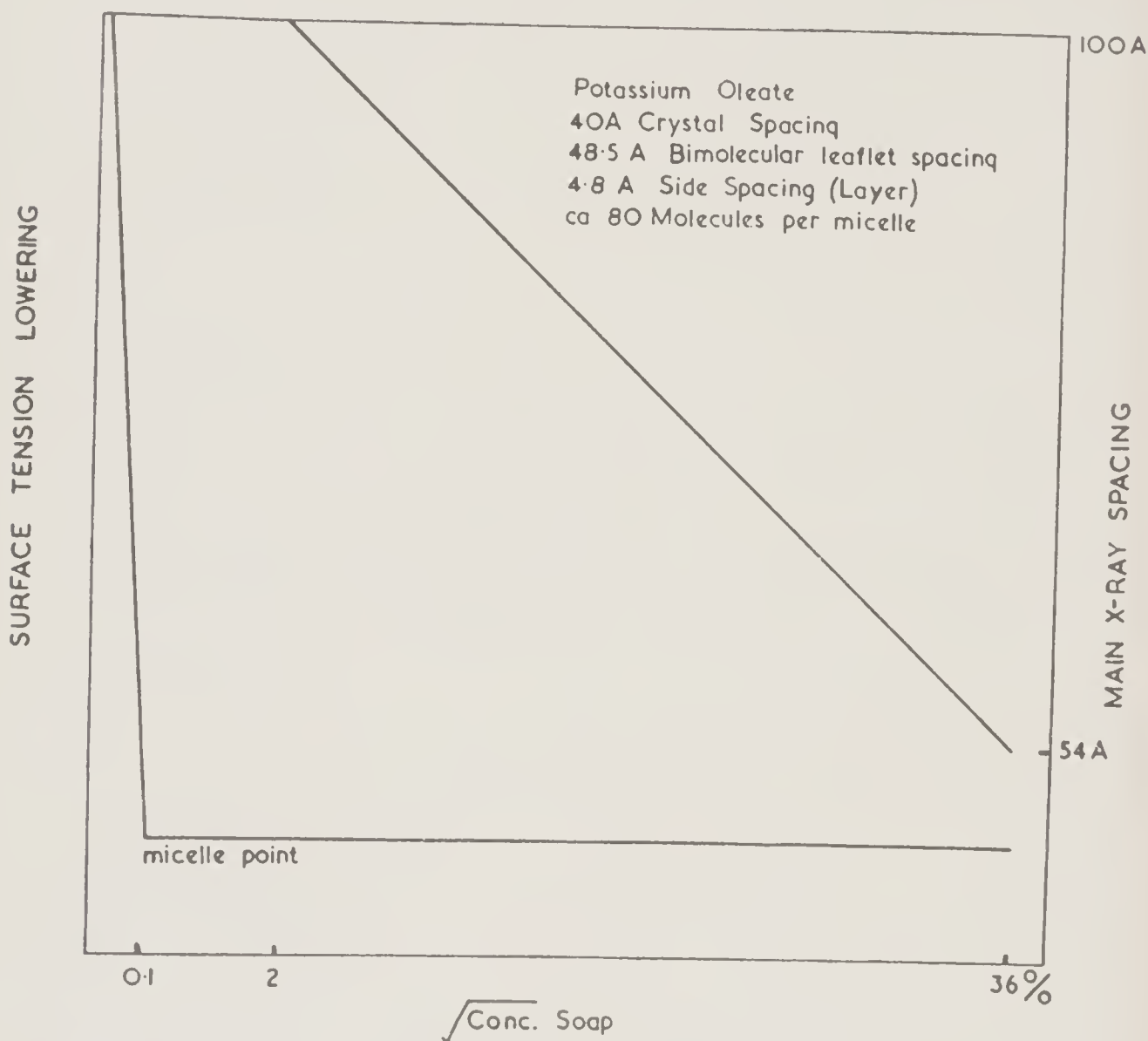
In Text-fig. 3 it can be seen that on increasing the soap concentration, the surface tension falls until a concentration is reached at which the surface monolayer is saturated with soap molecules in equilibrium with the bulk concentration according to Gibbs's Law. It can be readily understood that on further increase of the bulk concentration the molecules would associate in the form of micelles, leaving the surface concentration or surface tension constant. At this point, therefore, the conductivity and viscosity of the soap system will also change owing, in the first example, to some ions of opposite charge being adsorbed on to the micelle and migrating with the micelle in an electric field; in the second case it is due to the formation of streaming birefringent laminated structures by the associating molecules.

c. Penetration and Swelling of the Micelle Monolayer Lattices by Polar and Non-Polar Oils in Soap Micelles

By a combination of surface chemical, X-ray, and light-scattering techniques³⁴, accurate information can be obtained as to how polar or non-polar oil molecules can associate with soap micelles, and build up large aggregates. Detailed information can also be obtained on the structure of these systems, made up of large aggregates of heterogeneous composition.

It has been shown in the section on film penetration that before penetration of the monolayer takes place, strong polar association is

necessary between the polar groups of the film-forming molecule and the molecule in the underlying solution, together with a certain non-polar association³⁸. If the energy of association between the two molecules is greater than that of the film-forming molecules, no ejection of the penetrating molecule takes place on compression of an equimolecular



TEXT-FIG. 3. Relation between the structure of soap solutions and the surface tension as measured by X-ray diffraction measurements.

mixed film. If the polar association is weak, ejection of the penetrating molecule readily takes place. A striking similarity can be seen on the addition of a polar oil to a soap solution above the critical micelle concentration. If, from film-penetration work, the polar-oil molecule is known to associate with the soap molecule in the form of a two-dimensional complex, the polar-oil molecule will penetrate between the molecules in the two monolayers forming the micelle³⁶. This doubles the number of molecules forming the micelle, and thus enlarges the micelle laterally²⁵. No increase is obtained, therefore, in the X-ray scattering of either the bimolecular leaflet dimension or of the main large scattering

band^{25, 18}. On the other hand, the latter can be diminished by ejection of water between the micelle since, on penetration, the mixed monolayer is usually more hydrophobic in balance than the soap monolayer alone.

If there is no association between the polar molecule and the micelle-forming molecule, or the added molecule is completely non-polar, no penetration of the micelle monolayer takes place. This results in a swelling of the micelle lamellar, with an increase in the large X-ray scattering dimension. Thus it is shown that the non-polar oil has gone in between the two monolayers, or between the CH_3 groups at the end of the non-polar portion of the two film-forming molecules. (See Text-fig. 2 for mixed crystallite and oil- and water-swollen lamellar micelle.) The water molecules which can swell the lamellar and increase the distance between the polar portion of the film-forming molecules, is a similar occurrence. Typical examples are shown by the penetration of long-chain alcohols into the monolayer of the lamellar micelle of the soap, as compared to the swelling of the lamellar micelle by hydrocarbons such as benzene, paraffins, &c.^{36, 40}.

A more striking case is given by the chlorinated hydrocarbons. These do not form associations with carboxylic soaps, and hence can only swell the micelle in the presence of a polar oil, which can penetrate the soap monolayer. The entry of the chlorinated hydrocarbon, such as chloroform or carbon tetrachloride, is thus then permitted. On the other hand, chlorinated hydrocarbons possessing a labile hydrogen atom, as in chloroform or penta-chlor-ethane, can form complexes with nitrogen containing cationic soaps, such as C.T.A.B., long-chain pyridinium, or long-chain amine salts. These compounds will readily penetrate the micelle monolayer of the positive soaps, whereas substances possessing no labile hydrogen, e.g. carbon tetrachloride, cannot. The significance of this for emulsion formation will be demonstrated later²⁸.

d. Emulsion Droplet Formation from Soap Micelle

It can be seen that the soap micelle, both from the nature of the X-ray diffraction patterns and the fact that it only swells in a very limited manner in the presence of hydrocarbons, is not liquid in all directions. It can be shown that a combination of penetration of the soap monolayer by a complex-forming polar oil, and the swelling of the lamellar lattice by a non-polar oil, produces an aggregate which is liquid in all directions. Owing to the very low interfacial tension these liquid aggregates will be spherical. It can also be seen from Text-fig. 2 that the penetrated mixed lamellar can open out by occluding either oil or water, making droplets of oil in water or water droplets in oil. Starting from a system containing about equal proportions of water and a non-polar oil, and a concentration of soap in the water phase well above the

micelle concentration, a very fine fluid transparent emulsion can be obtained by simply titrating the disperse system with a polar oil, which must be able to form a complex with the soap molecules. In this emulsion the droplets of either oil or water can be as small as molecular dimensions. The transparency is due to the fact that the droplet diameters are below the $1/4$ wave-length of light and, as the droplet diameter approaches 500 Å, scattering of a Tyndall type takes place³⁶. The measurement of the intensity of the scattered light, as will be shown later, provides a way of measuring the size of the droplets.

As one may imagine, the systems made up of the lamellar micelle are streaming birefringent, whereas the penetrated and swollen spherical micelles give no birefringence at all, even with systems as concentrated as 40 per cent. of soap in the water phase. The control as to whether the resultant emulsion system contains water droplets in oil or oil droplets in water is governed by the wettability of the mixed interfacial film with oil or water. If the interfacial film is more wettable by water, water will be the continuous phase; and equally, if it is more wettable by oil, oil will be the continuous phase. The balance of wettability to oil or water, as will be shown later, is governed by the ratio of CH_2 groups to polar groups in the mixed monolayer. This balance is extremely sensitive, such that oil/water dispersion made up of amyl alcohol-sodium oleate will give a benzene/water transparent emulsion. The system hexyl alcohol-sodium oleate gives a water/benzene transparent emulsion (in high soap concentrations)³⁶.

Determination of the Size and Packing of Mixed Monolayer Stabilized Oil or Water Aggregates by Low-angle X-ray and Light-scattering Methods. X-ray Techniques^{34, 40}.

The X-ray diffraction bands obtained from soap solutions above the micelle point have been interpreted as Bragg spacings, answering the formula

$$n\lambda = 2d \sin \phi.$$

(X-ray deflexion angle ϕ for wave-length λ for the order n where d is the dimension of a molecular lattice repeat unit). Some doubt can be expressed as to whether these results mean diffraction from lattices, or possibly diffraction giving the distance between the centres of the aggregates or crystallites, which have a uniform ordered array. Where the micelles have been liquefied in all directions by the penetration of a polar oil, and swollen by a non-polar oil, the X-ray diffraction bands have been interpreted as giving the mean distance between the centres of the liquid aggregates. From a knowledge both of the molecular side spacings and of the area per double molecule of the mixed interfacial film obtained from surface chemical techniques, a calculated dimension

of the diameter of the oil or water droplets can be obtained from the simple formula:

$$r = \frac{3 \times \text{volume dispersed phase}}{\text{area of interfacial film}} = \text{Radius of oil or water droplets.}$$

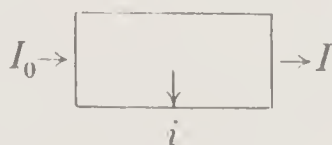
Further, knowing the ratio of the volumes of the continuous phase and the dispersed phase, calculations can be made on determining the distance (g) between the droplets. If l is the average length of the soap molecule in the mixed interfacial layer, then $2r + 2l + g$ should compare with the X-ray diffraction dimension obtained by multiplying the Bragg spacing with a factor 1.23, to compensate for a liquid as compared to a crystalline system.

These comparisons have been made for both the oil/water and water/oil systems, with the diameters of the droplets made to vary by changing the soap/alcohol concentration. This has shown that the low-angle X-ray method can justify the concept obtained by physico-chemical knowledge that the fluid transparent oil and water dispersions, made by a soap and polar-oil complex formation, are formed of close-packed uniform oil or water droplets. Each droplet is surrounded by a mixed monolayer of the complex soap/alcohol. Dimensions up to 800 Å for the diameters of the spherical micelle have been measured by the low-angle X-ray technique, Text-fig. 4 (no correction is given for the gap between droplets for calculated and observed diameters).

In order to change the distance g (the space between the droplets), which can be calculated from the geometry of the system, a dilution series of the droplets in the disperse or continuous phase can be made. This could be followed by the increased dimension given by the changing X-ray diffraction band. The calculated and observed values agree well.

*Light-scattering Techniques*³⁴

It has been shown that the X-ray scattering technique gives information relating to the mean distance between two centres of the aggregates. Thus, in order to determine the aggregate size by this technique, they must be close-packed and uniform. It is interesting to note that with the light-scattering technique the diameter of the aggregates can be measured but not the distance between them, and then only if the concentration of the aggregates is dilute, and if the diameter is small (at least 16 times smaller) in comparison to the wave-length (λ) of the incident light. The relationships between the intensities of the incident light (I_0), transmitted light (I), and right-angle scattered light (i),



when the incident light is polarized, are given by:

$$\frac{I}{I_0} = e^{-\tau x}$$

where τ is the turbidity per cm. x or

$$\frac{i}{I_0} = \frac{9\pi^2 N V^2}{\lambda^4 D^2} \left(\frac{n_1^2 - n_2^2}{n_1^2 + 2n_2^2} \right)^2$$

Rayleigh equation.

N = number of particles; V = volume of droplet;

n_1, n_2 = refractive index of each phase;

D = distance of observer from cell,

or
$$H \frac{c}{\tau} = \frac{32\pi^3 n^2 (dn/dc)^2 c}{3\lambda^4 N_0} \frac{c}{\tau} = \frac{1}{M} + \frac{2Bc}{RT}$$

derived from the considerations of Smoluchowski and Einstein²⁷.

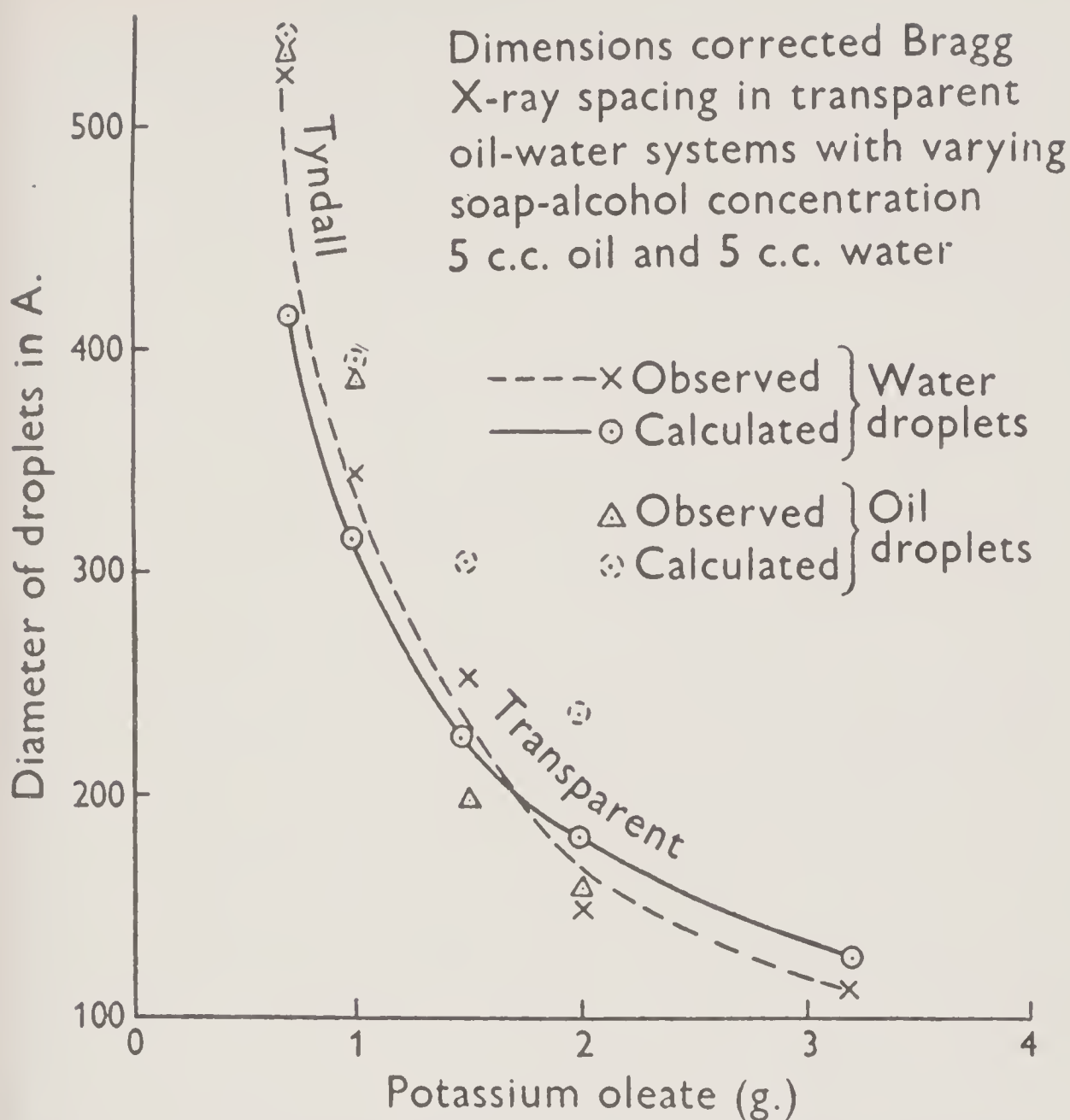
c = concentration of solute; M = molecular weight solute;

R = gas constant; B = co-volume factor;

N_0 = Avogadro number; n = refractive index solution.

The Rayleigh equation is derived from the consideration of the energy taken up by a gas molecule from the incident light beam, and then scattered in all directions. The Smoluchowski and Einstein equation is derived somewhat differently. They considered that the intensity of scattered light depended on local concentration and density fluctuations, in a solution or pure liquid respectively, brought about by thermal activity in the solution. Thus the Rayleigh equation can only be applied to comparatively dilute dispersions where the absorption of light is very low and, if monochromatic visible light is used, up to dimensions of about 400 Å diameter of the particles. The Einstein equation can be conveniently applied to the determination of the molecular weight, size, and shape of macro-molecules or polymers, over a considerable concentration range. In high protein concentrations, though, deviations from the law are also observed.

The intensity of the right-angle scattering, in relation to the incident light intensity, has been measured for the transparent oil and water disperse systems, and Rayleigh's equations applied to the results³⁴. These results were compared to the calculated dimensions of the system of water droplets surrounded by a mixed monolayer of *p*-methyl cyclohexanol and potassium oleate in benzene. They were further compared to the X-ray data on the diameter of the water droplets for the same system. It was found that Rayleigh's equation gave a diameter for the droplets very close to the calculated one, over the range 50 Å diameter to 400 Å diameter, if the system of water droplets was diluted progressively in the benzene continuous phase (and the result taken at infinite



TEXT-FIG. 4. Diameter of droplets calculated from area of soap molecules as established by surface chemical techniques in comparison with diameter of droplets measured by X-ray diffraction.

dilution). This was necessary to overcome the difficulty of multiple light absorption in the cases of close-packed spheres. Rayleigh's equation gives the product (NV^2) , number of particles multiplied by their volume squared. Since the product NV is known from the quantity of material in the incident beam, the volume, and thereby the radius of the particle, can be determined. It can easily be seen visually that in dilute systems where the droplets are of the order of $500 \text{ \AA}$, a strong Tyndall effect prevents the application of the Rayleigh equation for the wave-length of visible light.

The fluid transparent oil/water dispersions, therefore, are excellent material for a quantitative investigation by X-ray, surface chemical, and light-scattering techniques (and possibly electron microscope), of the structure, spacing, and dimensions of aggregates over the range

50–1,000 Å. In this range of dimensions the ordinary microscope cannot assist. The above techniques have enabled a detailed investigation to be carried out on the behaviour of soap micelles during their penetration by polar oils, their swelling by non-polar oils, and the increase in droplet diameter on decreasing the monolayer area at the oil/water interface. The way is now open for an application of this work to biological systems, composed of the same type of compounds in fine dispersion.

Hydrophobic and Hydrophilic Balance of Interfacial Films in Relation to Phase Continuity in Emulsions

There are three rules governing phase continuity in emulsions. These seem to hold for all systems of stabilizing agents and for different oils. It must be remembered that the interfacial film counts as a phase, besides the oil and water phases.

1. If the interfacial film is charged by adsorption of positive or negative ions, or consists of anionic or cationic long-chain hydrocarbon stabilizing agents present in dilute salt solution, then a diffuse ionic concentration gradient can be built up in the aqueous phase at the interface. This gives the droplet a zeta potential. Since the ionic gradient can only be held in high dielectrics, the charged droplets of the same sign will repel one another. Under these conditions it is necessary for the droplets to be oil and the continuous phase water. If this ionic concentration gradient be removed by addition of salts, or chemical means such as the tanning of cationic soaps with polyphenols, &c., one obtains an ion pair dipole or phenolic amine complex dipole, which answer to rule 2 or 3.

2. If the interfacial film of the stabilizing agent, or agents, be more soluble in oil than in water, then oil will be the continuous phase. This is also the case if the contact angle with oil or water for a monolayer of the stabilizing agents be greater with oil than with water at the oil/water interface.

3. If the reverse is the equilibrium condition, so that the interfacial film be more soluble in water, or the contact angle made by such a film with oil and water at an oil/water interface be greater with water than with oil, then water will be the continuous phase.

If a single compound is used to stabilize an emulsion, then the oil or water solubility of that compound can give direct information as to which phase will be the continuous one.

Examples

Interesting examples of this latter class are the non-ionogenic compounds, such as the condensation products of the long-chain alcohols with ethylene oxide¹⁰. In water-wetting power the oxygen atom equals three

CH_2 groups in balancing out their oil-wetting power. One ethylene oxide group already containing two CH_2 groups equals one CH_2 group in the alcohol radical chain. Thus $\text{CH}_3(\text{CH}_2)_6\text{O}[(\text{CH}_2)_2\text{O}]_3\text{H}$ will give water/oil emulsions, and $\text{CH}_3(\text{CH}_2)_6\text{O}[(\text{CH}_2)_2\text{O}]_5\text{H}$ will give oil/water emulsions.

If two stabilizing agents are used, one of which is an oil-soluble agent, and the other a water-soluble agent, the phase continuity is governed by the nature of the monolayer formed at the interface. There are at least four possibilities. If no complex is formed between the two agents either the oil-soluble or water-soluble component will sit alone at the interface by displacing the other, according to the magnitude of the interfacial lowering properties of either agent. This is controlled by their mutual oil or water concentrations and their chemistry, which governs their mutual adhesion and cohesion to the oil or water phase.

If there is complex formation between the two stabilizing agents, it is usual for the equimolecular mixed monolayers resulting from the association to be more stable than the monolayer of either of the two components alone. This results in very low interfacial tensions, small droplet size, and spontaneous forming emulsions. The hydrophobic/hydrophilic balance of the mixed monolayer must now be considered, in order to establish which phase will be continuous—similar to the considerations given for the penetrated and swollen soap micelle.

*Oleophobic Surfaces*³¹

A special case of interest is a surface that is not wettable by either oil or water. Such a surface is obtained by adsorbing long-chain polar compounds on to a base in which the polar group adheres strongly, or where the polar groups are bound together in double layers. This ad-lineates the CH_2 groups in the hydrocarbon chain in such a way that the end CH_3 group attached to each chain is tightly arranged in the form of a crystal monolayer lattice. The CH_3 surface cannot associate with the free CH_2 group or other hydrocarbon groups in any deposited oil. Contact angles of 42° with metals coated with long-chain amines having chain length of at least $(\text{CH}_2)_{14}$ have been obtained with hydrocarbon oils⁶. Any disorder brought into the CH_3 lattice array immediately permits the oil to spread, as on normal paraffin wax.

*Intestinal Fat Emulsions*¹³

It can be shown that digestion of a triglyceride such as triolein by pancreatin is auto-inhibiting, since the oleic acid produced by the hydrolysis will prevent protein from reaching the fat molecules at the interface. The soap will deterge the protein from the oil/water interface. The addition of calcium ions will remove the soap, permitting digestion of the glyceride to recommence. The monoglyceride formed by the digestion can be shown, from force/area curves, to pack best at the

interface, displacing the triglyceride and oleic acid, thus making a very low interfacial tension. Since the molecule is oil soluble, however, the hydrophobic/hydrophilic balance will favour the formation of an oil-continuous water in oil emulsion. Oleic acid formed *in situ* at the interface will, in solutions more acid than pH 8.5, also favour water/oil emulsions. Above pH 8.5, where no hydrolysis of the sodium oleate can take place, the surface will become negatively charged through the negative carboxyl ion, and the emulsion will invert to oil/water. Since environment conditions in the duodenum necessitate an emulsion stable to pH conditions of at least pH 6 to 8.5, this double system of oleic acid monoglyceride is therefore not sufficient. It is known that oleic acid can form a complex with the bile salts, such as sodium taurocholate, which possesses a negative sulphonate ion dissociated over the whole range of acid or alkali solutions. This bile-salt negative ion can, in acid solution, be hooked into the mixed monolayer of monoglyceride-oleic acid by complex formation. It will also ionize the interface in acid solutions, and thus, according to rule 1, invert the emulsion back to an oil/water system. The triple system—monoglyceride for interfacial tension lowering; oleic acid for complex formation in acid solution with the bile salt; and the bile salt, producing an ionic concentration gradient at the interface—enables oil/water emulsions to form. This system now possesses all the criteria necessary to produce fine oil/water emulsions under the biological conditions of the intestine. These negatively charged emulsion particles, of $\frac{1}{2}\mu$ or less, can now be made to migrate under a membrane potential negative to the lumen side, through a porous membrane, of $\frac{1}{2}\mu$ pore size, under a concentration gradient C_1/C_2 of sodium taurocholate. The mobilities of the sodium (u) and taurocholate (v) are different (sodium having the greater).

Thus the membrane potential $\mu = \frac{u-v}{u+v} \frac{RT}{F} \log \frac{C_1}{C_2}$ and the ratio of the concentration of the negatively charged oil particles, inside C_3 and outside C_0 , of a membrane, will be governed by the expression

$$\frac{C_3}{C_0} = e^{-E\mu/(RT/F)}$$

(F = one Faraday; E = charge of particle.)

In this case charged particles will be made to migrate through the membrane against their own concentration gradient. It can be seen from the logarithmic expression that small changes in membrane potential, or charge on the oil droplet, will produce large changes in the concentration of negatively charged particles on either side of the membrane³⁰.

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CHAPTER IV

THE CELL SURFACE AND CELL PHYSIOLOGY

By J. F. DANIELLI

i. INTRODUCTION

IT is not intended here to cover all the ramifications of this subject, but rather to deal particularly with those branches in which there have been substantial advances in the last twenty-five years (i.e. since Bayliss wrote the last edition of his *General Physiology*)² and also with certain other branches in which many of the preconditions for such an advance appear to exist. During the course of this chapter it will become clear, if it is not already so, that there is a hiatus between the cell surface and the bulk of the cytoplasm and that the relationships between events occurring in these two parts of the cell are but little explored. In this respect a difficulty and gap in our knowledge occurs, analogous to the situation in nerve physiology some years back, when it was known that an impulse in one nerve axon on reaching a synapse elicited a response in a second axon, but the mechanism of transmission across the synapse was unknown.

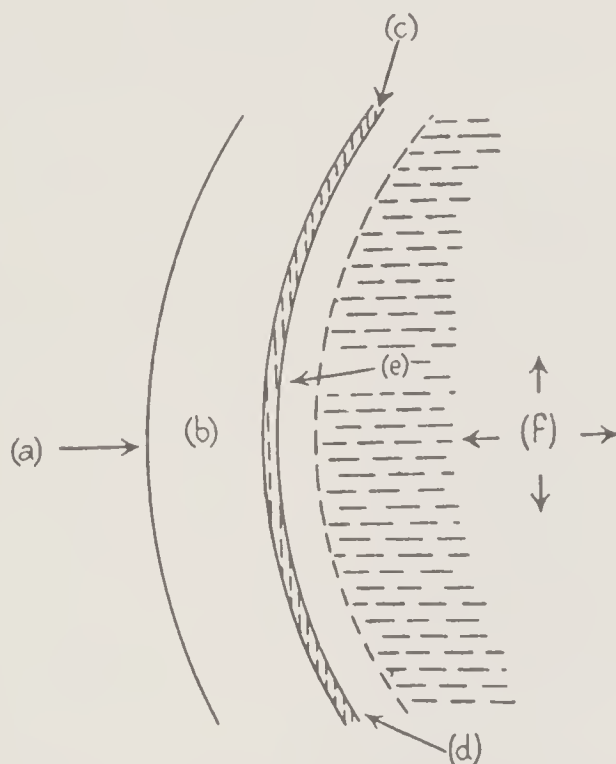
When considering the cell surface it is the region reaching inwards from the plasma membrane with which we are concerned. Many cells have supporting or protecting membranes outside the plasma membrane, but micro-dissection has shown that these outer membranes are not essential for life, whereas the plasma membrane cannot be removed, or even badly damaged, without death of the cell. We must therefore regard the plasma membrane as a part of the cell as essential as, for example, the nucleus. Reaching inwards from the plasma membrane is a more or less narrow band, the ectoplasm, which is usually gelled and relatively free from cytoplasmic granules, &c. (Text-fig. 1). In many ways this layer is closely similar to the bulk of the cytoplasm, but it is also directly concerned in, for example, cell division, and so comes into the picture of the cell surface also.

ii. STRUCTURE AND PHYSICAL PROPERTIES OF THE PLASMA MEMBRANE

a. Molecular Structure

The great majority of cells studied have a common ground-plan for the structure of their plasma membranes which we are now able to describe, though our description is crude and includes no detail and no consideration of local differentiation of the membrane. In this group we can include with certainty such cells as erythrocytes, nerve axons,

muscle fibres, leucocytes, yeast, *Nitella*, many cells of higher plants, and marine ova such as those of sea-urchins and starfish. On the other hand, permeability measurements suggest that the sulphur bacterium *Beggiatoa mirabilis* has a membrane of rather different structure. Virus particles differ from cells in having no plasma membrane and are



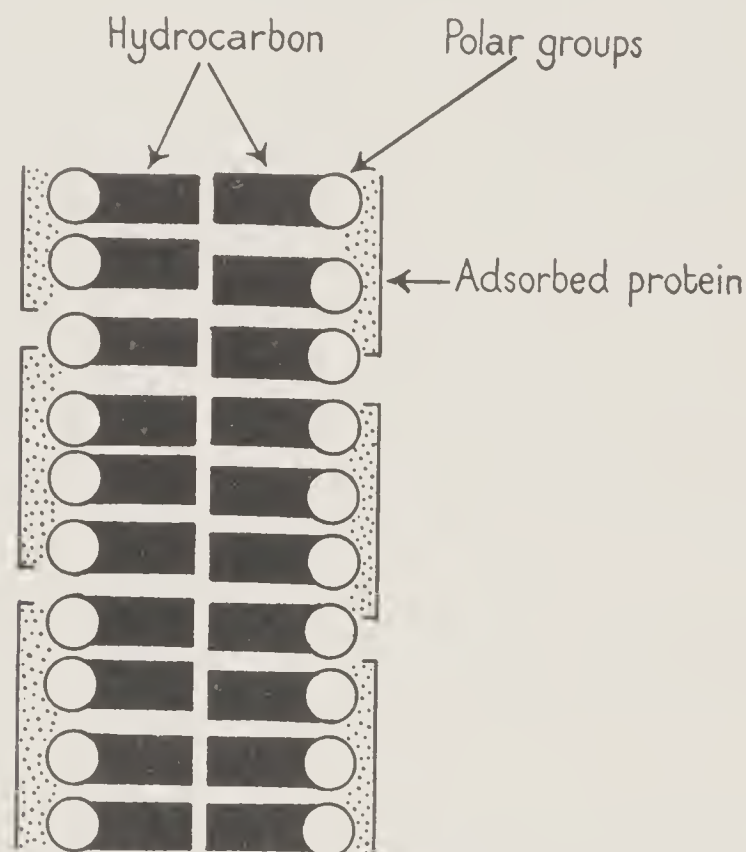
TEXT-FIG. 1. Diagram of surface layers of a fertilized *Arbacia* egg.

a. Fertilization membrane. b. Perivitelline space. c. Hyaline plasma layer. d. Plasma membrane. e. Gelled cortex or ectoplasm. f. Endoplasm.

therefore unable to control their internal composition in the same way that cells do.

The three fundamental properties of the membranes of the main group of cells, from which we proceed to define the membrane structure, are (1) preferential permeability to lipid-soluble substances, (2) the existence of a low tension at the surface, (3) high electrical resistance. Properties (1) and (3) indicate that there must be a continuous layer in the membrane composed of lipid molecules, such as phosphatides, sterols, and fats. Property (2) indicates that at the surfaces of the membrane there is an adsorbed protein layer. The arrangement shown in Text-fig. 2 is the simplest possible structure which can meet these essential requirements. It consists of a continuous film of lipid molecules, of which the two outermost layers are so orientated that the hydrated polar groups are in the oil/water interfaces, with a layer of protein molecules adsorbed on both of these interfaces. It cannot yet be said to what extent the protein is mechanically superimposed upon the liquid,

and to what extent the surface must be regarded as a specific protein-lipoid complex.* With this arrangement of molecules the tendency of the lipoid molecules to change their orientation will be insignificant, and since the membrane is a continuous surface (a spherical shell) all surface-tension forces will balance out and will not tend to disrupt the film.



TEXT-FIG. 2. Diagram of molecular structure of the plasma membrane.

Spherical shell membranes of this type can be made experimentally and show considerable stability.

Various other arrangements of the lipoid molecules in a continuous lipoid film are conceivable, all of which, however, are less satisfactory, since they both tend to revert to the type of Text-fig. 2, and also to coagulate irreversibly the proteins adsorbed upon them. These cases are discussed in detail elsewhere (Danielli, 1936¹⁷).

The adsorbed layers of protein shown in Text-fig. 2 are the primary adsorbed layers, consisting of denatured (possibly reversibly denatured) protein. The protein in these layers consists of polypeptide chains, or meshworks of such chains, lying in the plane of the interface, with the hydrocarbon portions of the amino-acid residues dissolved in the lipoid layer and the polar groups in the aqueous phase. There may be a further layer of globular protein adsorbed on to this primary layer; it is almost certain that this will be so in all cases on the inner (cytoplasmic) surface of the membrane. Since the adsorption of globular proteins is readily

* See Chapter III.

reversible, the presence of globular proteins on the outer surface will depend on the nature of the bathing fluid; when this is plasma, cells usually behave as though coated with globulin.

The mechanical properties of the adsorbed polypeptide chains are probably largely responsible for the elasticity and relatively great mechanical strength of the plasma membrane. A single polypeptide chain may be $50\text{ m}\mu$ or more in length and will be attached to similar chains by hydrogen bonds and other linkages and will thus 'tie' the various parts of the membrane together, but, owing to the elastic properties inherent in such interlinked chains, without conferring upon the membrane an undesired brittleness.

That the cell surface is a lipid layer was first suggested by Overton, as a result of his work on cell permeability, but difficulties in the interpretation of permeability studies soon led to a suggestion from Nathanson that the cell membrane is really a mosaic of areas of different properties, some adapted to permit passage of one type of molecule, others to permit passage of quite different molecular types. This suggestion has fluctuated in popularity, but evidence is accumulating to show that whilst the continuous lipid layer described above is the basic pattern of the cell membrane, there are relatively small areas which are specially differentiated, so that we are beginning to adopt Nathanson's suggestion in a somewhat modified form. These special areas or patches, in some cases at least, are comparable with the active patches of a catalyst: for example, Jacobs and his colleagues have found that certain erythrocytes are abnormally permeable to glycerol, but that acidity, or a minute trace of copper, will reduce the permeability to a value typical of most other cells. From the kinetics of penetration it also follows (Danielli³²) that the areas permitting abnormal permeation are a small fraction only of the total membrane area. One of the chief tasks before workers on cell permeability is to discover the extent, structure, and function of these specialized patches.

A further counter-suggestion to Overton's hypothesis was that the cell membrane is a molecular sieve, containing water-filled pores, through which molecules of diameter less than the pore diameter would diffuse quite readily. For most cells this theory is now discredited, the only exception being that of *Beggiatoa*, for which diffusion kinetics suggest that the molecular-sieve hypothesis is correct. This, however, is still by no means certain³².

b. Thickness

The thickness of the plasma membrane has been calculated by four methods. Gorter and Grendel⁴¹ have found that the total lipid extractable by lipid solvents from the erythrocytes of various species would make a layer not more than bimolecular in thickness. Consequently, in

the case of the erythrocyte there is a clearly defined upper limit to the thickness (but not a lower limit). It is possible that Gorter's choice of solvents has led to a low value, but not more than 50 per cent. low.

The second and third lines of approach are from the impedance of cell suspensions. Fricke³⁷ has shown, from the capacity of the cell membrane, that the thickness of the erythrocyte wall is of the order of 50 Å; Fricke and Curtis have obtained a similar value for polymorphonuclear leucocytes³⁹ and for yeast cells³⁸. Cole's results^{11,12} with the eggs of *Arbacia*, *Asterias*, and *Hippoonoe* also give values of the same order. However, as these authors have pointed out, the analysis leading to this conclusion is far from unequivocal. The agreement between Fricke and Gorter and Grendel has been considered to place a satisfactory upper limit of 2–4 molecules to the thickness of the lipid layer in erythrocytes. It is improbable that the values obtained for the other cells are in error by a factor of more than tenfold.

From the resistance of the cell membrane it can be calculated that the thickness lies between 1 μ and 1 m μ .

The fourth line of approach is that of Schmidt *et al.*^{89,90}, who conclude that the red cell envelope consists of a few layers of radially orientated lipid molecules and a number of tangentially orientated protein polypeptide chains. These results were obtained from birefringence studies.

From these investigations it appears improbable that any cell yet studied has a lipid layer thicker than 10 m μ .

c. Ionic Character

The fatty molecules adsorbed at the two oil/water interfaces of the membrane are oriented so that their polar groups will be directed, on the average, with the positive end of their electrostatic dipoles towards the oil layer and with the negative end towards the aqueous phase. Owing to this orientation of electrical dipoles, ions from the surrounding aqueous phase will distribute themselves, negative ions inside the oil layer around the positive ends of the dipoles, positive ions in the aqueous phase around the negative ends of the dipoles. Thus there will be an excess of negative ions inside the oil layer (Davson and Danielli³⁰). The nature of these ions may exert an influence on the structure and behaviour of the membrane, though the evidence available suggests that this influence is small compared with that of the cations in the aqueous phase.

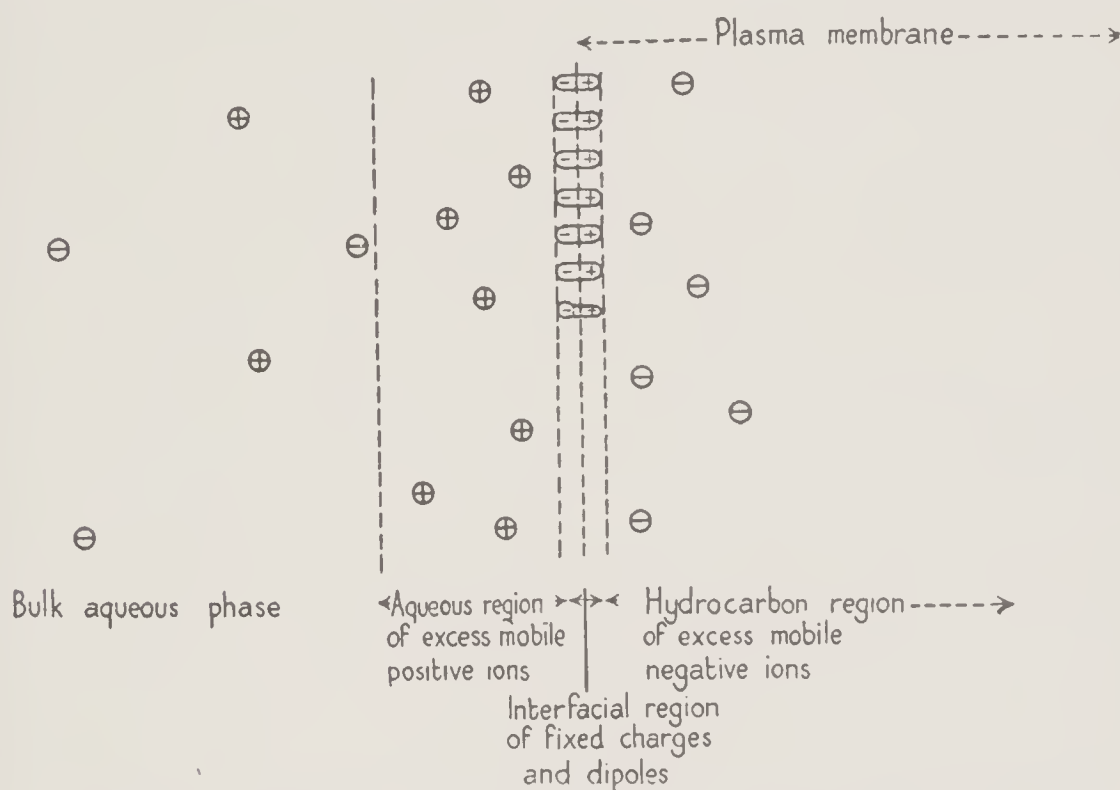
In the aqueous phase will be certain cations distributed round the negative ends of the dipoles of the polar molecules, and also other cations distributed around the carboxyl* and phosphatide* ions in the surface. Thus, in the aqueous phase in the immediate vicinity of the cell membrane there will be (a) an excess of mobile positive ions over mobile

* Attached to the lipins and proteins.

anions and (b) an excess of cations over the concentration in the bulk aqueous phase bathing the cell (Text-fig. 3). The thickness of this region of excess ions may be calculated from the theory of Debye and Huckel³⁴ (1923). At 20° C. in solutions of uni-univalent salts the thickness is

$$\delta = \frac{3.1 \times 10^{-8}}{\sqrt{C}} \text{ cm.}$$

For $C = 0.1$ molar, $\delta = 0.98 \text{ m}\mu$, and for $C = 0.0001$, $\delta = 31 \text{ m}\mu$. Thus in dilute solution this region of excess ions may be as thick or



TEXT-FIG. 3. Diagram of electrical structure of the plasma membrane.

thicker than the lipid layer itself. The concentration of the various ions at the surface of the cell may be calculated from the Gibbs-Donnan equilibrium (Danielli^{18,25}) or from the electrokinetic potential (Hartley and Roe⁴⁵). At the surfaces of most cells the ratio, average concentration of univalent cations in surface layer:concentration of cations in normal environment, lies between 1.1 and 5. Since this applies to hydrogen ions as well as K^+ , Na^+ , &c., the pH at the surface of a cell is usually between 0.1 and 0.7 pH units different from that of the medium. The inverse rule applies to mobile anions; there will be a deficiency of mobile anions in the aqueous layer adjacent to the membrane, so that the ratio, average concentration of univalent anions in surface:average concentration of anions in normal medium, lies between 1/1.1 and 1/5.

It is theoretically possible that locally the excess of cations in the surface may exceed the values indicated by the ratios just given. In artificial systems values as high as 200 have been found for this ratio (Webb and Danielli⁹⁹). Wilbrandt¹⁰⁰ has pointed out that when divalent

ions are considered the excess concentration in the surface becomes even more marked, for it follows from the Gibbs-Donnan equilibrium that the relationship between the univalent and divalent ion concentrations is that

$$\text{if} \quad \frac{[\text{Na}]_{\text{surface}}}{[\text{Na}]_{\text{bulk}}} = r,$$

$$\text{then} \quad \frac{[\text{Ca}]_{\text{surface}}}{[\text{Ca}]_{\text{bulk}}} = r^2.$$

Hence the ratios for calcium concentrations for cells in their normal environment lie between $1 \cdot 1^2$ and 5^2 , or, i.e. between 1.2 and 25, according to the cell. Webb and Danielli⁹⁹ showed that the surface excess of total calcium in the surface may exceed the values calculated in this way, for the calculation just given refers to *ionic* calcium only, and in addition calcium may be held in a surface in an apparently non-ionic form. Owing to the operation of these two factors it is found that at the surface of most cells, when the external medium contains ions giving a Na:Ca ratio of about 100:1, the calculated ratio in the surface may be roughly 1:1 (Danielli²⁴). This may be a very important fact, for the work of Ringer, Loeb, and many others has shown that for a cell to behave normally it must be bathed by a solution containing a Na:Ca ratio of the order of 100. Usually a type of cellular behaviour dependent on the cell surface is at one extreme in solutions containing NaCl only, at another extreme in CaCl_2 , and a balance between the two is achieved when $\text{Na:Ca} \rightleftharpoons 100$. It now seems possible that the reason for this value for the bulk ratio is that this bulk ratio is what is necessary to give a ratio of *c.* 1 in the surface, where the balancing action has to occur. It is quite impossible for one Ca ion to counterbalance 100 Na ions, but when the ratio is near to unity this difficulty vanishes. Some caution must be observed in using this theory, for at present the evidence that the surface ratio is close to unity is indirect, based on calculations and not direct experimental evidence.

d. Lysis

There are innumerable ways in which lysis may be brought about. Many lytic agents cannot penetrate the cell membrane, so that, with such agents, lysis must be the result of direct action upon the cell membrane. In these cases, as Davson²⁹ suggests, we are carrying out a partially controlled degradation of the cell membrane, somewhat analogous to degradative analysis in organic chemistry. The most significant types of lysis, from our present point of view, are produced by (a) antibodies, (b) polyhydroxylic phenols, (c) heavy metals, (d) lipoid solvents, (e) lecithinase, (f) digitonin, (g) sodium or potassium salts of fatty acids and paraffin sulphonates $\text{C}_n\text{H}_{2n+1}\text{SO}_4\text{Na}$. The first two of these reagents

probably act almost exclusively on the protein part of the membrane and the latter four mainly on the lipid part of the membrane. Heavy metals probably act on both lipoids and on proteins.

Antisera to cells will contain antibodies to the adsorbed protein on the cell surface, and it is presumably to these that the lytic action is due, as it is very doubtful whether antisera to lipoids can be made. The antibodies, by combining with the membrane proteins, will break down other complexes existing between the protein and the membrane lipid molecules, and so produce a general disruption of structure. The polymerized polyhydroxylic phenols exert a tanning action on protein films (Schulman and Rideal⁹¹) and consequently will also disrupt the structure in much the same way. The simple polyhydroxylic phenols may act in a similar way (Davson and Danielli³¹), but it is difficult in such cases to be sure that the lytic action is not due to the slow formation of polymers. The lytic action of these two types of substances which attack protein primarily shows the great importance of the primary adsorbed protein layer.

Heavy metals probably act by combining with the carboxyl and phosphate groups of the proteins and lipoids, to give non-ionic compounds, and probably also causing closer packing of certain types of molecules than is normal. It is most interesting in this connexion that Jones⁶¹ has found that the toxic action of the heavy metals is proportional to their solution pressure, i.e. the more readily a metal forms unionized complexes with anions, the greater is the toxic action of the metal.

Of the remaining lytic agents, lipid solvents probably act by dissolving in the lipid layer and breaking down its structure by solvent action; lecithinase by converting lecithin into lysolecithin (which is itself lytic); digitonin by forming a complex with the membrane sterols; and the salts of fatty acids and paraffin sulphonic acids by forming complexes with the sterols, &c., though it is possible that they also react with the protein layer. Schulman, Rideal, and their colleagues have shown that reactions of the last three types will all occur in monolayers.

Thus the action of these reagents for lipoids shows that the lipid part of the membrane must also retain its normal structure within comparatively narrow limits, if the membrane is to remain stable.

e. Surface Tension and Elastic Properties

The surface tension of cells, more properly called the tension at the surface, has frequently been invoked to explain cell division. The tension at the surface is defined as the sum of the elastic tensions and surface tensions of the plasma membrane. There are two ways in which surface tension may affect cell division. (a) A belt of the surface in the cleavage plane of a cell may have its surface tension raised; the result

would be that the inwardly directed pressure in the vicinity of the cleavage plane would exceed that elsewhere, so that cleavage would occur if the rise in tension were sufficient. But it is very difficult to believe that this can occur. In the first place, during cleavage the cortical gel layer in many species has marked rigidity, so that a very considerable rise in tension would be required to cut through the cortex. In the second place, local differences of tension in an area less than 1 sq. cm. vanish in a fraction of a second, whereas cleavage may take many minutes, so that for division to result from local tension changes it would be necessary to provide for continuous localized production of a substance capable of raising the tension at a lipo/protein surface by many dynes; there are many reasons for regarding this as improbable. Thirdly, the cell contains, and is usually bathed by fluids containing, many substances of great surface activity which should prevent such rises in tension occurring. (b) Alternatively, division may occur if the cell were elongated, by, for example, growth of the spindle, to a length $\pi(3.14)$ times the diameter of the cell, since such a cylinder divides spontaneously, without any local changes in tension being necessary. But Table I shows that in fact cells divide long before they have elongated to this extent, so that this alternative can be immediately discarded.

TABLE I. *The ratio of the long axis (l) to the short axis (b) at the time of initiation of the cleavage furrow in various cells. Spontaneous division would occur if $l/b \geq \pi$.*

Cell	l/b	Authority
Egg of <i>Echinus esculentus</i> in sea-water	1.5	Gray (1924) ⁴²
Ditto in Ca-free sea-water	1.9	Gray (1924) ⁴²
Egg of <i>Arbacia punctulata</i>	1.4	Just (1939) ⁶²
Cell from choroid of chick's embryo	2.0	Strangeways (1922) ⁹⁶
Spermatocytes of <i>Hemiptera</i>	1.35	Bowen (1920) ⁴

Further light has been thrown on this problem by direct measurements of the tension at the surface of cells. Early attempts at such measurements were invalidated by experimental errors, and the successful techniques are mainly due to E. N. Harvey, K. S. Cole, and their colleagues. Many techniques have been used,* of which the centrifuge method, the compression method, and the kinetic method are typical. The centrifuge method may, for example, be applied to marine eggs in which, under centrifugal force, a stratification of the cell contents occurs, the lighter parts moving to the centripetal end of the egg and the heavier parts to the centrifugal end. When the separation has occurred the difference in density between the two ends of the egg gives

* See review, Harvey and Danielli⁴⁹.

rise in a centrifugal field to forces elongating the egg into a cylinder, which divides into halves when its length exceeds π times its diameter. Then, knowing the minimum centrifugal force necessary to obtain sufficient elongation and the density difference between the two halves, we can calculate the force required to elongate the egg, and equate this to the tension round the circumference of the elongated cylinder. For the unfertilized *Arbacia* egg the tension at the surface found by this method is 0.2 dynes/cm., an extremely low value (Harvey⁴⁶).

Cole¹⁰ compressed *Arbacia* eggs with a minute gold beam. From the pressure exerted by the beam he was able to calculate the internal pressure of the egg (due to the surface tension) and from this to calculate the surface tension. He obtained a value of 0.08 dynes/cm. for the uncompressed egg by extrapolation of the tension-compression curve to zero pressure. The membrane was definitely elastic. Harvey and Danielli⁴⁸ studied some of the types of surface systems which might occur in the plasma membrane, finding that all surfaces containing protein, and only surfaces containing protein, have elastic properties. Cole's demonstration of elasticity in the plasma membrane may therefore be regarded as supporting the view that protein is adsorbed on the cell surface.

Sichel and Burton⁹⁴ showed that the tension at the surface of the blastomeres formed by the first cleavage of *Arbacia* eggs has a similar value, 0.09 dynes/cm. They followed the cleavage of the egg until the blastomeres were connected by a small stalk only, then punctured one of the blastomeres and took moving pictures of the decrease in volume of the other blastomere as its contents discharged through the stalk. From the rate of discharge, assuming Poiseuille's law held, the excess pressure inside the egg, due to surface tension, was calculated; from this pressure the surface tension was derived. Evidence was also found of elastic properties of the cell surface which may, however, in these measurements be due to other causes.

Another method, from Harvey's laboratory, is to stretch a cell between two micro-needles and calculate the tension from the force necessary to obtain a given degree of elongation of the cell. Norris⁸³ obtained values of the tension of the surface of *c.* 0.1 dynes/cm. for *Arbacia* eggs and of less than 0.1 dynes/cm. for erythrocytes.

Results for a number of different cells are shown in Table II. The tension at the surface is always lower than 2.0 dynes and often less than 0.2 dynes/cm. These low tensions seemed at first incompatible with the view that the cell membrane is a thin lipoid layer, as natural lipoids have much higher tensions against sea-water than, for example, the 0.1 dynes per cm. found for *Arbacia* eggs (actually 0.05 dynes at each side of the membrane). However, Harvey and Shapiro⁵³ showed that intracellular oil droplets in fish eggs also had very low surface tensions and had elastic surfaces—as has been more recently shown for oil

droplets in *Amoeba proteus* and in eggs of the fresh-water crustacean *Daphnia pulex* (Harvey and Schoepfle⁵²)—and Danielli and Harvey²⁷ showed that the substances responsible are proteins, which by adsorption on the lipid surfaces both reduce the tension at the surface to extremely low values and also render the surface elastic.

It will be seen that these studies of the tension at the cell surface have been most useful in developing our knowledge of the structure of the cell membrane.

TABLE II. *The tension at the surface of various cells*

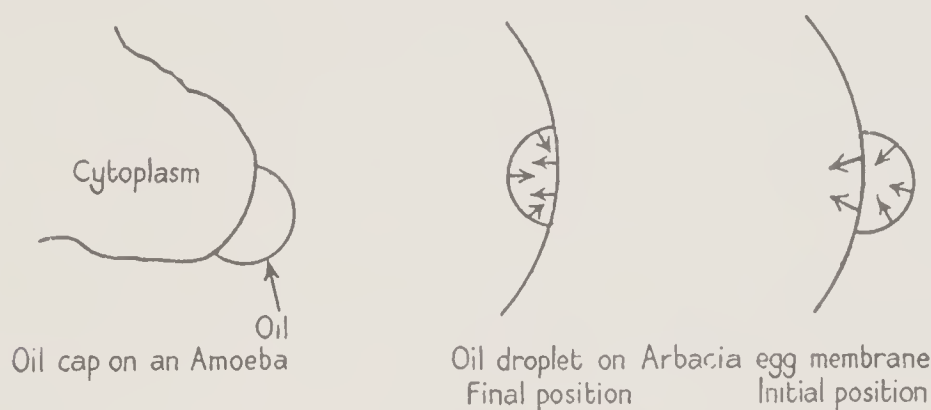
Cell	Tension dynes/cm.	Authority
<i>Arbacia punctulata</i> : egg . . .	0.2	Harvey, 1932 ⁴⁶
	0.08	Cole, 1932 ¹⁰
	0.09	Sichel and Burton, 1936 ⁹⁴
	0.1	Norris, 1939 ⁹³
<i>Triturus pyrrhogaster</i> : erythrocyte	0.1	Norris, 1939 ⁹³
<i>Amoeba dubia</i>	1.3	Harvey and Marsland, 1932 ⁵¹
<i>Physarum polycephalum</i> . . .	0.45	Vexler, 1935 ⁹⁸
Rabbit macrophage	2.0	Shapiro and Harvey, 1936 ⁹³
Frog leucocytes	1.3	Shapiro and Harvey, 1936 ⁹³
<i>Busicon caniculatum</i> : egg . . .	0.5	Harvey and Fankhauser, 1933 ⁵⁰
<i>Triturus viridescens</i> : egg . . .	0.1	Harvey and Fankhauser, 1933 ⁵⁰

f. Wetting Properties

Some very interesting results have been obtained by endeavouring to cause oil to coalesce with the cell membrane. Mudd and Mudd^{79,80} investigated the ease with which various types of cell are wetted by, and coalesce with, a film of oil advancing between a cover-slip and slide. Erythrocytes were readily wetted by the oil, but leucocytes have a hydrophilic surface, and when engulfed by an advancing film of oil retain a thin layer of saline between the cell membrane and the oil. An oil droplet in saline is readily wetted by such a film of oil, but if protein is added to the saline the surface of the droplet becomes hydrophilic and such a droplet behaves like a leucocyte. Consequently Mudd and Mudd suggested that leucocytes are coated with protein, whereas erythrocytes have a surface layer of lipid material. From what we now know of proteins at the oil/water interface it can be said that the surfaces of both cells are probably coated with protein, but that, while the outermost layer at the surface of leucocytes is a strong elastic gel layer, probably serum globulin, the layer at the surface of erythrocytes is probably a weakly elastic or liquid layer, possibly of serum albumin, which is known to form comparatively fragile films. In this connexion it is interesting to find that Ponder and Furchgott⁸⁷ have found that maintenance of the discoid form of the mammalian erythrocyte is connected

with the adsorption of a crystallizable fraction of the serum albumin upon the erythrocyte. Reversible disk/sphere changes occur, according to whether this albumen is, or is not, present in the fluid bathing the erythrocyte.

Dawson and Belkin³³ found that a globule of oil readily adheres to the surface of *Amoeba dubia*, forming a cap (Text-fig. 4). The cap is not drawn into the interior of the cell. Marsland⁶⁸ used this technique to



TEXT-FIG. 4. Diagrams illustrating the behaviour of oil droplets brought into contact with the cell surface. The arrows indicate the internally directed force in the droplets due to interfacial tension.

investigate the site of narcosis by paraffin oils. The oils were diluted in olive oil and the solutions either injected into the interior of an *Amoeba* or fixed on its surface as a cap. At a sufficient dilution of paraffin with olive oil the injected droplets had no harmful effects on the *Amoebae*, but the caps still caused clearly defined narcosis. It seems beyond doubt that the site of narcosis in this instance is the cell membrane and that the paraffin dissolves in the lipid layer of the surface, as other lipid solvents do. Chambers⁸ and Kopac and Chambers⁶³ have obtained equally convincing evidence with naked *Arbacia* eggs from which the pellicle has been removed. Here an applied droplet of a suitable oil 'snaps in' when applied to the surface, making a flattened surface of contact on the inside of the cell membrane, and *lifting the membrane off the granular cytoplasm*. By directing a stream of water from a micro-pipette against the cell surface the membrane can be made to flow, and if two such 'snapped in' droplets are adhering to the membrane, they can be seen to move relative to one another. It seems incontestable that the plasma membrane of *Arbacia* eggs is fatty or lipid in composition and, moreover, is liquid.

The difference between *Amoeba dubia* and the egg of *Arbacia* seems to depend upon whether the ectoplasmic or cortical layer, underlying the plasma membrane, is a gel or not. If it is a rigid gel, capping occurs. If it is liquid or a weak gel, penetration occurs, owing to the surface tension outside the membrane being greater than that inside the cell. The surface tension is lower inside owing to the high concentration of proteins. With *Arbacia* penetration occurs, for example, only if the

interfacial tension of the penetrating oil is greater than about 9.5 dynes/cm. Thus, on touching the cell surface in protein-free sea-water the surface tension at the outer surface of the oil drop is about 9.5 dynes/cm., whereas adjacent to the ectoplasm it is about 0.05 dynes. Hence the internal pressure of the droplet due to the external interfacial tension will be, for a drop of 5μ in radius, about 4×10^4 dynes/cm.², and the interfacial pressure due to the side adjacent to the ectoplasm is only 2×10^2 dynes/cm.² This difference in pressure literally forces the drop through the plasma membrane. The critical tension of 9.5 dynes, giving a pressure of 4×10^4 dynes/cm., probably represents the pressure necessary to deform the cortical gel, lift the plasma membrane off the gel, and permit entry of the droplet. If the sea-water contains protein it adsorbs on the oil, the surface tension is reduced, and penetration cannot occur.

These experiments of Kopac and Chambers are particularly valuable, since they give that direct proof of the existence of a lipid layer at the cell surface in one case which many investigators have regarded as so essential. Many lines of indirect evidence have pointed to this conclusion for many different types of cell. This direct proof for the *Arbacia* egg enables us to utilize the indirect evidence available with other cells with much more confidence than would otherwise be the case.

g. Permeability

In considering the permeability of the cell surface we must initially divide the field into two sections, the first dealing with thermal diffusion which involves no expenditure of energy by the cell, the second dealing with assisted diffusion, or secretion, in which substances are redistributed wholly or partly as a result of cellular activity involving the utilization of energy. The first section can now be dealt with quantitatively, to a first approximation, but secretion is a field in which we are ignorant, even of the outline of the processes involved.

The permeability of cells to non-electrolytes is a function of the character both of the cell membrane and of the penetrating molecule. The more important molecular characteristics involved are molecular weight and diameter and the nature, polar or non-polar, of the individual groups of the molecule. It is the balance between polar and non-polar groups which determines the oil/water partition coefficient of a molecule. Overton, in the last decade of the nineteenth century, found that permeability was a function of the oil/water partition coefficient of the penetrating molecules, so that molecules with a high oil/water partition coefficient are able to penetrate more rapidly than molecules with a small partition coefficient. This led Overton to suggest that the cell membrane is a thin lipid layer, and although many alternative hypotheses have been considered, for the great majority of cells for which we have experimental results this theory of Overton's is un-

doubtedly correct. A possible exception is *Beggiatoa mirabilis*, the membrane of which may be a molecular sieve containing little lipoid. But, as has been remarked above, special areas of certain cells are differentiated to permit penetration of certain particular molecules, so that the lipoid layer is certainly not in all cases a complete homogeneous layer. Table III shows the permeability of the membranes of different cells to ethylene glycol, glycerol, and erythritol, compared with the permeability of a roughly equal thickness ($5\text{ m}\mu$) of water. For such molecules the permeability of the cell membrane is at least 10^9 times less than the permeability of the same thickness of water. Very few physiologically important substances have a permeability greater than 10^{-8} of that of the water layer, significant exceptions being oxygen and carbon dioxide. It is most remarkable that a membrane only $5\text{ m}\mu$ in thickness should be able to bring about this reduction in diffusivity. It has been calculated (Danielli¹⁹) that a hundredfold increase in thickness would make comparatively little difference to the permeability of the cell membrane to many molecules, such as glycerol and glucose; the optimum efficiency is secured by a layer only a few molecules thick, and further increase in thickness would be a comparatively expensive luxury.

TABLE III. *Calculated permeability of a thin oil layer and experimental values of the permeability of some cells. Permeability in mols./sec./ μ^2 per mol. per litre concentration difference, $\times 10^{16}$*

	5 m μ water, viscosity = 0.01 poise	5 m μ oil, viscosity = 10^3 poise	Ox erythro- cyte	Arbacia egg	Chara cerato- phylla	Plagio- thecium denticu- latum	Pylaiella litoralis
Ethylene glycol	1.7×10^9	0.1	0.2	0.73	1.2	0.03	0.1
Glycerol	1.4×10^9	0.002	0.002	0.005	0.02	0.0003	0.002
Erythritol	1.2×10^9	0.00005	..	..	0.001	0.00007	0.0001

Table III also shows that there is a considerable degree of specificity, different cells having different permeabilities to the same molecule.

Experimental results with ions show that here, also, there is considerable variation from cell to cell, and even in the cases of greatest permeability to ions the cell membrane still reduces the permeability to a value not more than 10^{-8} of that of a water layer of the same thickness. Generally speaking, the smaller, i.e. the less hydrated, an ion the more readily it penetrates the cell membrane. Maizels⁷⁰ has shown that the permeability of erythrocytes to different anions is $\text{NO}_3 > \text{Cl} > \text{acetate} > \text{oxalate} > \text{sulphate} > \text{citrate}$: this is the order of increasing hydration. With regard to cations, it is known that with most cells the small ions potassium, rubidium, and caesium can penetrate much more rapidly

than the large ions lithium and sodium. Davson²⁸ has shown that similar considerations come into play when simultaneous diffusion of both anions and cations is studied.* With regard to specificity: it is generally considered that erythrocytes are much more permeable to anions than to cations, and that most other cells, including muscle and nerve cells, are more permeable to potassium than to anions. In the view of the present writer, however, it is probable that most cells will be found to be comparatively readily permeable to small anions such as Cl^- , and that in addition many cells are also permeable to potassium and to a much less extent to sodium; the failure to observe anion permeability with, for example, muscle cells, in the case of ions such as Cl^- , is probably due to the equilibrium conditions determining the amount of Cl^- in the muscle cell being such as to practically exclude Cl^- from the interior of the cell. This view is supported by the fact that phosphate penetrates muscle fibres quite readily.

There is little doubt that many of the more important facts about permeability and secretion of ions remain to be discovered. This process will be assisted by the use of isotopes, with the aid of which it is possible to study the gross transfers of ions from one side of a membrane to another, and so determine the actual rate of movement of the different ionic species. The net transfer of ions is the most that can be determined without the use of isotopes. However, it is important to note that the most important recent individual advance in our knowledge of ionic movements, due to Harris and to Maizels, was achieved without the use of isotopes. By the ingenious control of experimental conditions these workers have demonstrated that movements of sodium and potassium into and out of the human red cell are to a large degree dependent upon glucose metabolism, that sodium is actively extruded from red cells, and potassium may perhaps be taken up by an active process.

When we turn to large molecules, such as proteins, sterols, and neutral fats, we find that in many cases penetration of the cell membrane does occur, but calculations show that penetration cannot occur by thermal diffusion (Danielli²¹). One can only conclude that such molecules enter the cell through specially differentiated areas, by special mechanisms, of which we are at present ignorant. A detailed review of the permeability of cells to ions and molecules is given by Davson and Danielli³¹.

The temperature coefficient of penetration of most molecules and ions is of the order of 2 or more per 10°C . rise in temperature. As diffusion in water has a Q_{10} of 1.2 to 1.4, these large Q_{10} values were at one time supposed to be due to transport across the cell membrane being dependent upon the mediation of a chemical reaction. We know now, however, that these large Q_{10} values may arise in simple diffusion processes:

* There is evidence that the results in such cases are complicated by the phenomenon of 'anomalous' osmosis.

where diffusion is fast, the resistance to diffusion is small and the Q_{10} is small. Where diffusion is slow, the resistance to diffusion is large and the Q_{10} is large. These conclusions follow from a simple consideration of diffusion kinetics. The study of the kinetics of diffusion across a thin lipid layer has shown that in all qualitative and in many quantitative aspects thermal diffusion is able to account for the various rates of penetration of different molecules, and that only where penetration is shown to be associated with the utilization of energy by the cell need special chemical reactions be invoked. From these studies of diffusion kinetics it has been found possible to calculate the approximate permeability of a thin lipid layer. Some results of such calculations are shown in Table III for glycol, glycerol, and erythritol. The order of magnitude agrees well with that found for living cells.

The cell membrane presents three sites of resistance to free diffusion: (1) the membrane/water interface, for diffusion from water to membrane; (2) the membrane/water interface, for diffusion from the membrane into water; (3) the interior of the membrane. Every molecule penetrating into the interior of the cell has to pass through these three sites of resistance. A molecule with polar groups in it, e.g. hydroxyl (OH) groups, as opposed to, for example, non-polar groups such as methylene (CH_2), forms at least one hydrogen bond with water for each polar group. All these hydrogen bonds must be broken simultaneously if a molecule is to penetrate into the (lipoid) membrane, since otherwise the molecule is not free to diffuse into the lipid layer. Thus glycerol, having three OH groups, must acquire sufficient kinetic energy to break three hydrogen bonds simultaneously before it can penetrate into the membrane. This involves a large amount of energy; consequently resistance (1) is so high for glycerol that resistances (2) and (3) are dwarfed into insignificance. On the other hand, a molecule like methyl alcohol, having one OH group and one CH_3 group, can easily penetrate into the membrane and can also easily pass out of the lipid layer into water. Thus we find that for such molecules the rate of diffusion across the interfaces is large compared with the rate of diffusion across the membrane, i.e. the viscous resistance (3) of the interior of the membrane is the most important factor in controlling penetration. Differing from both of these examples are predominantly hydrocarbon molecules, such as carotene, $\text{C}_{40}\text{H}_{56}$. For such a molecule resistance (1) is insignificant, for the molecule has no polar group, and even resistance (3) is not enormous, but resistance (2) is very large indeed. This is because hydrocarbon groups are hydrophobic and a considerable amount of kinetic energy is required to transfer a CH_2 group from lipid into water: when many such groups are present they must all be transferred simultaneously from the lipid layer into water, since otherwise the molecule remains substantially part of the lipid layer and cannot diffuse away into the aqueous

phase. So when a molecule contains many non-polar groups resistance (2) becomes of major importance. With these examples in mind we can classify penetrating molecules into four groups:

- (a) Molecules having few polar and few non-polar groups: for these resistance (3) is most important; they usually penetrate comparatively rapidly, e.g. oxygen, methyl alcohol.
- (b) Molecules having a predominantly polar character: resistance (1) is most important; penetration is slow, e.g. glycerol, sugars, glycogen.
- (c) Molecules having few polar and many non-polar groups: resistance (2) is the most important; penetration is slow, e.g. carotene, vitamin A, cholesterol, fats.
- (d) Molecules having many polar and many non-polar groups: resistances (1) and (2) are both important; penetration is slow, e.g. polyhydroxylic bile acids, the glucuronide of oestrin, proteins.

These relationships between different molecular species and the cell membrane are of great importance, both for the life of a single cell and for the life of the larger organisms. Let us consider just a few of these applications. Oxygen is required in large amounts by the cell, and CO_2 must be disposed of rapidly. These substances penetrate the cell membrane rapidly. On the other hand, the first products of glucose utilization by, for example, a contracting muscle cell, such as glycerol derivatives, are valuable: the cell membrane does not allow these to escape. But during sustained work lactic acid is formed: this would be toxic if allowed to accumulate, but the cell membrane is comparatively permeable to lactate, so that lactic acid can escape into the blood. This allows violent exercise to be maintained for a period perhaps 50 per cent. longer than would be the case if the cell membrane were impermeable to lactic acid. Turning then to substances to which the cell membrane is impermeable, we find the problem of storage of vital materials is solved by the utilization of the properties of certain types of molecules—amino-acids are stored as protein, fatty acids as neutral fat, and sugar as glycogen; in each case, as a result of polymerization, a substance is formed which cannot penetrate the cell membrane. An alternative use of inability to penetrate the cell membrane is seen in detoxication mechanisms, in which toxic substances are conjugated with amino-acids, or with sulphuric acid or glucuronic acid, so that a toxic cell-penetrating substance such as brombenzene or menthol is converted into a new molecule which cannot penetrate into cells and which, once reaching the blood-stream, is bound to be filtered off by the glomerulus of the kidney, and will not be resorbed from the urine in the tubules unless it happens to fit into the rather specific secretory mechanism of the tubules. Thus by conjugation with a molecule such as glucuronic acid two objects are secured simultaneously: (a) the toxic substance is

prevented from penetrating into cells, and (b) its excretion in the urine is secured.

Turning now to consider very briefly the problems of secretion, it is possible that at least two seats of activity can be distinguished, the cell surface and the cell interior. Many theoretical proposals have been put forward, involving one or both of these sites, by which molecules such as sugar and ions such as amino-acids and potassium may be moved against a concentration gradient. But the most that can be said for these proposals is that whilst they are theoretically possible, very few of the assumptions have yet been subjected to experimental examination. Experimental work on the mechanism of secretion is scanty and mostly of two types, either exploration of the mechanical factors involved or the use of enzyme poisons to see how the source of energy may be cut off. As an example of the first type we may consider the contractile vacuole: it is possible that all the work is being done during the swelling phase of the vacuole, in which unwanted substances are collected from the cytoplasm and separated from other cytoplasmic constituents. Ejection of fluid may result mechanically from the vacuole coming into contact with the plasma membrane; since the vacuole has a higher curvature than the plasma membrane it will have a higher internal pressure than the rest of the cytoplasm if both membranes have the same tension at the surface; consequently, if the vacuole membrane fuses at any point with the cell membrane and the cell membrane cannot achieve the same curvature as the vacuole membrane, the excess internal pressure will force the vacuole contents out into the medium, just as, in the case discussed under 'Wetting Properties', an oil drop of sufficiently high surface tension may be forced into the interior of a cell. Enzyme poisons such as cyanide, fluoride, iodoacetate, and phloridzin are frequently used in the study of secretion, with the object of finding the particular enzyme systems concerned. But at present it is very difficult to avoid the argument that such poisons may block secretion by interfering with the metabolism at a point far removed from the secretory apparatus. By analogy one may consider the investigation of the process by which an electric lamp produces light; one may cut off the light by dropping a bomb on the power station or by removing the filament from the lamp. At present we can only drop the bomb, whereas what we need to do is to examine the filament.

h. The Ectoplasmic Layer

In his book on the cell surface Just⁶² gives a stimulating account of much of what is known of the ectoplasm of the cell. Although this is achieved at the expense of largely ignoring the cell membrane, Just has performed a timely service in his discussion of a field that has of recent years been somewhat neglected. By the ectoplasm we refer to the

cytoplasmic region lying immediately inside the plasma membrane. Its most prominent characteristics are its gelled condition and its relative freedom from the various cytoplasmic formed bodies which constitute a large part of the more fluid, more central endoplasmic region. The ectoplasm is not to be confused with other gel-like structures outside the plasma membrane: these can usually be washed away, or dissected away, without grave consequences. The ectoplasm, on the other hand, is an essential part of the cytoplasm, and its partial differentiation from the endoplasm is partly due to the presence of the plasma membrane. In most cells the ectoplasm or cortex is a relatively rigid gel and is comparatively clear. It may be liquefied reversibly. For example, Heilbrunn and Daugherty⁵⁵ found that K^+ decreases the viscosity, or rather rigidity, of the ectoplasm of *Amoebae* and at the same time increases the viscosity of the endoplasm. The reverse action is found with Ca^{++} and Mg^{++} . These observations suggest that K^+ partly releases a gel-forming substance from the cortex and that the divalent ions cause it to be more concentrated in the cortex. Heilbrunn believes the substance involved is ionic calcium. It seems more probable to the present writer that the action of the ions is to lower (in the case of K^+) or raise (in the case of Ca^{++}) the value of the resting potential across the plasma membrane, and that this decreases or increases the rigidity of the gel by decreasing or increasing the orienting action of the cell membrane on the myosin-type molecules involved in gel formation. Marsland and Brown⁷³ found that high hydrostatic pressure will also reversibly liquefy the ectoplasmic gel. Just suggests that this clear gel is substantially identical with the clear ground-substance of the endoplasm, the differences in physical properties and behaviour being comparatively minor. This suggestion seems quite plausible when it is remembered that the endoplasm also is the site of continuous gel $\rightleftharpoons$ sol changes, and it is possible that the main distinction between the ectoplasm and the endoplasmic ground-substance lies in the localization of more gel-forming material in the ectoplasm—a localization which may be due entirely to the cell membrane. This continuity of structure is rendered the more probable by the absence of any clear division between ectoplasm and endoplasm: the two appear to merge in most cells by quite imperceptible stages, the gel simply becoming weaker at greater distances from the cell membrane.

Sol $\rightleftharpoons$ gel transformations in the ectoplasm are most important in amoeboid activity, in cell division, and probably in protoplasmic streaming. It is also possible that the formation of connective tissue fibres, cartilage, and bone is an activity of the ectoplasm.* The intercellular

* X-ray studies (see review, Picken⁸⁵) show that many tissue fibres consist of parallel cross-linked polypeptide chains. Astbury has shown that somewhat similar fibres may be made artificially from protein monolayers. Hence it is worth

bridges known to occur in plants, and believed to occur in animal tissues, are probably mainly ectoplasm. The fine tentacles and filaments thrown out from the surfaces of many cells owe their stability to the rigidity of the ectoplasm. The ceaseless streaming movements of the plasma membrane, so beautifully shown by Chambers's films of marine ova, may be due to the movements of the ectoplasm. Just suggests that muscle cells consist mainly of ectoplasm and that it is to this that muscle owes its high contractile power. Mirsky⁷⁶, from studies on isolated myosin, has concluded that the myosin in muscle must be in a gelled condition.

Various types of further differentiation of the ectoplasm are found in special cases; for example, the brush border of intestinal cells, flagella, cilia, contractile vacuoles, &c. Wherever visible movements occur, the ectoplasmic gel, or some analogous gelled structure, such as the mitotic spindle, is involved. How far the organized molecular movements involved in secretion are made possible by gel structures remains to be seen.

i. The Physiological Response to a Stimulus

The cell membrane is not merely a boundary between the cytoplasm and the environment and a convenient support to some enzyme systems. It is also the receptor system for all stimuli which are not in the form of radiant energy, coming from the environment or from other cells (really also part of the environment). It is also the nearest point of approach to other cells, so that it is from the cell membrane that stimuli are given out to other cells. At, for example, a synaptic junction of two nerves it is the adjacency of the membranes of two nerve cells, the property of one membrane to emit acetylcholine and of the other membrane to respond to acetylcholine, which enables a stimulus to be conveyed from one cell to the next. The nature of the response to a stimulus is therefore a matter of great importance. R. S. Lillie^{64,67} has discussed the possibility that the initial response to a stimulus is an increase in permeability, applying this theory very widely, from the response of a nerve to electrical excitation to the response of an egg to sperm entry. In the case of nerve, Lillie's theory is now known to be substantially correct. There is a potential difference between the two sides of a resting nerve membrane—the resting potential—which may be reduced by an artificial potential difference applied in the opposite direction. According to Lillie's theory, when this reduction is sufficiently substantial the permeability of the nerve membrane is increased; adjacent charged regions of the membrane are then able to discharge through the more permeable area, as a result of which the newly discharged region becomes more permeable; thus a region still farther away from considering whether these fibres are not spun off from the adsorbed film of protein present at the surface of cells.

the initial point of discharge is enabled to discharge through this newly permeable region. This process, under appropriate conditions, should continue indefinitely, each region losing its charge by discharge through a more permeable region slightly nearer to the site of the initial stimulus. So the response to a stimulus consists in a discharge of the resting potential followed by an increase in permeability: the conduction of an impulse consists of a wave of discharge, followed by a wave of increased permeability. And the recovery process, from the physical point of view, consists in the restoration of the membrane potential and permeability to their initial values. That discharge of the resting potential does accompany transmission of the impulse was, of course, known for many years, and was one of the main facts on which Lillie based his theory. A. V. Hill and his colleagues measured the heat evolution accompanying transmission of the impulse, finding it to be so very low that it is hardly possible for the impulse to involve more than a minor physical change in the membrane.* Hodgkin⁵⁸ was able to demonstrate that the local electrical currents postulated by Lillie's discharging process do actually exist, and Cole and Curtis¹³ have shown that a large increase in permeability does follow closely the discharge of the resting potential. On the other hand, even after the increase in permeability has reached its maximum, the nerve membrane retains a very considerable resistance, so that only minor changes in structure can have occurred.

Thus far, attempts to understand the detailed molecular mechanism of the conduction of the nerve impulse have been few, and the only mechanism so far suggested which is compatible with other knowledge of the cell surface is that of Danielli, who suggests that the permeability changes are due to changes of state in the lipid layers of the plasma membrane. These changes of state are attributed to the changes in the electrical potential gradient across the membrane. The theory appears to be compatible qualitatively with all the available facts about the conduction of electrical impulses, and has not yet been studied quantitatively. A factor which remains to be considered in this, as in many other fields, is the possibility that the membrane structure may be sufficiently organized for some of the electrons of the molecules concerned to occupy levels characteristic of the membrane as a whole. If this is so, the membrane must to some degree be a conductor for electrons. Originally this type of view was suggested by Szent-Gyorgi as a possible approach to the problem of muscular contraction, but it clearly may be of interest in many fields, including the properties of the cell surface.

Turning to the other extreme, the response of eggs to activation, Lillie was able to show that following activation, either by sperm or by parthenogenic reagents, *Arbacia* eggs show an increase in permeability

* The recovery heat, evolved long after the impulse has passed, is much larger and does involve chemical processes.

to water. On the other hand, Shapiro, for example, has shown that fertilization causes very little change in the permeability of *Chaetopterus* eggs, and Lillie found no change in the permeability of *Asterias* eggs after fertilization, so that if a permeability change is really responsible for initiating development, it must be comparatively transient. But Gray has pointed out that in artificial parthenogenesis it may be necessary to expose the egg to the activating agent for a considerable period, so that it may be necessary to maintain a condition of increased permeability for much longer than is the case with nerve and muscle for the stimulus to be effective. Lillie's theory, in its more extreme forms, must probably be rejected, in so far that stimulation of the ectoplasm may probably occur without a change in permeability, but in considering the immediate response of a cell to most forms of stimulation the evidence strongly favours his view that a change in permeability is of fundamental importance. Consequently one of the great problems confronting those who work on the cell surface is to discover the molecular mechanism whereby these reversible changes in permeability may occur.

Heilbrunn⁵⁴ has advanced a second theory of some importance, not necessarily in contradiction with that of Lillie, but rather intended to show how the stimulus, once received by the cell surface, causes a response which, as in the case of the muscle fibre, may involve the bulk of the cytoplasm. According to Heilbrunn, a stimulus causes release of calcium ions which had hitherto been bound to the proteins and lipoids of the cell surface or the cortical gel layer, and this released calcium initiates the further activity of the cytoplasm. Heilbrunn and his colleagues, particularly Mazia, have brought together a considerable mass of evidence to show that there is an increase in the ionic (ultra-filterable) calcium after stimulation. Unfortunately it is extremely difficult experimentally to distinguish between calcium released in *immediate response* to a stimulus, and calcium released as a *result* of activity initiated directly by the stimulus without the intervention of calcium ions. However, it seems far more probable, to the present writer, that calcium is released rather late in the process of response. Calcium ions can be bound, either in the ionic or non-ionic state, by combination with anions on the surfaces of colloidal molecules, such as proteins. They can therefore only be released by (a) the production of another cation which is preferentially bound, (b) by the production of acid (or some equivalent process)* which will reduce the number of ionizing acidic groups on the surface of the colloids, or (c) by the conversion of the colloids into smaller molecular units, for example, a protein into amino acids. Consequently a

* The same result would be achieved by, for example, decarboxylation of a protein, or intramolecular rearrangement in a protein molecule which resulted in bringing adjacent carboxyl groups closer together, reducing the probability of dissociation of the carboxyl groups.

stimulus, to release calcium, must first supply either alternative cations for binding, acid or its equivalent, or else initiate the degradation of colloidal particles. So that the chemical event of increase in the free calcium concentration can hardly be one of the earlier events in response: it is much more likely that it is in most cases a consequence of the production of acid (CO_2 , lactic acid) which is the invariable result of cellular activity.

These theories of Lillie and Heilbrunn are the only ones for which sufficient experimental evidence has been adduced to be taken seriously, and, as we have just seen, Lillie's theory is correct in certain cases, but the underlying molecular mechanisms are unknown; whilst Heilbrunn's theory has less evidence in its favour, and again the molecular mechanisms involved by such a theory have hardly been considered. It may therefore be said that the response of cytoplasm to a stimulus is still, on both the physiological and chemical levels, a most intriguing mystery.

Many workers, both biologists and chemists,* have felt that the co-ordinated activity of the cytoplasm demands the existence of a cytoplasmic skeleton, capable of linking the bulk of the cytoplasm with the cell surface and the nucleus. But all attempts to find this cytoplasmic skeleton have failed, and evidence has steadily accumulated to show that the cytoplasm is fluid and usually of low viscosity (see Chapter II). We are thus in a position somewhat analogous to those nineteenth-century physicists who sought in vain for a material and ponderable ether. But in the last few years a new possibility has appeared, as a result of studies made on gels and sols of tobacco mosaic virus and on gels of certain other materials, such as bentonite. As was pointed out in Chapter II, there are long-range electrical forces operating between large colloidal molecules. These forces are responsible for orienting long, needle-shaped protein molecules parallel with one another, and for some types of gel-formation. Thus undisturbed cytoplasm may have a structure based on orientation of molecules, without physical contact being necessary between the molecules concerned; the cell membrane, nuclear membrane, and other semi-permanent structures in the cell form an initial base with respect to which orientation may be reconstituted after disturbance. It is well known, for example, that the ectoplasmic layer is frequently gelled and is much more rigid than the regions farther away from the membrane. The stability of this gelled layer almost certainly depends on the state of the cell membrane, and there is no apparent reason why this orientation pattern based on the cell membrane should not extend into the bulk of the more fluid cytoplasm. It must be emphasized, however, that this is speculation, and at present we can do no more than consider the possibilities of such a theory. One

* See R. A. Peters's lecture, *J. State Medicine*, 37 (1929), as an example of the latter.

of the most attractive of these possibilities is found in connexion with muscular contraction. Birefringence studies show us that in a resting muscle-fibre the myosin molecules (the major protein constituent) are partly arranged as rodlets whose long axis is parallel to the long axis of the fibre. As a result of stimulation, the resting potential across the fibre membrane is greatly decreased and subsequently the fibre develops a tension; simultaneously the orientation falls off. How the fall in resting potential leads to contraction is unknown. But the conception of orientation in an electrostatic field offers a possible mechanism: let us assume that the myosin molecules nearest to the cell membrane are given a definite orientation by the potential across the membrane; since the fibre membrane has a large curvature except along its long axis, the orientation will be parallel to the long axis. These oriented molecules will then constrain other molecules to orientate in the same direction. The stability of this orientated system then depends entirely on the membrane potential, and if this is discharged the myosin will tend to revert to a state of more random orientation. As Meyer has pointed out, such a loss of orientation will be attended by decrease in length, i.e. by contraction. The process of contraction may, therefore, simply mean that, when the constraining influence of the membrane potential is removed, the molecules revert to a more probable state of orientation. As a result of such a reversion, heat will be evolved for just the same reason that stretched rubber, on contracting, evolves heat. The restoration of the resting potential then becomes an essential precondition for restoration of orientation, i.e. for relaxation.* However, whilst it is most desirable that the possibilities of electrostatic forces acting on colloidal molecules should be investigated as far as possible, it is unlikely that our first guesses about the role of these forces will be quite correct. Moreover,

* Whilst the first step in contraction may well be breakdown of orientation and organization due directly to the transient decline of the resting potential, the myosin molecules may actually pass through a much more complicated cycle of events than simply disorientation and reorientation. Possibly changes in the myosin micelles occur. Possibly, as Bernal has suggested, the orientated micelles in a resting muscle have a loose packing (analogous, perhaps, to tobacco mosaic virus gels) and in contraction a closer packing (possibly analogous to the virus crystals). To some extent supporting this view are the observations of Mirsky⁷⁴ on frog and rabbit limb muscle and of Danielli¹⁹ on crab limb muscle, in which it was found that a decrease in myosin solubility occurs after extensive periods of contraction or in rigor. Mirsky showed that these changes may also be produced in myosin preparations *in vitro* and are not analogous to denaturation by heat. Then there is the further question of the role of the chemical events involved in muscular activity: at present we do not know even which of the chemical changes known to occur are due to restoration of the resting potential and which changes participate more directly in the contractile process.

A change in solubility of a myosin-type protein also occurs after fertilization of *Arbacia* eggs (Mirsky), suggesting that this protein is involved in the mechanism of cell division and mitosis.

in the processes we have just considered we have still not covered the hiatus between the act of contraction and the chemical events involved. But at least it can be said that some such trigger mechanism as that just described does exist in muscle and that the cell surface is a very important part of this mechanism.

Equally provocative is the response of cells to drugs, such as adrenaline, histamine, and acetylcholine. Ability to respond to low concentrations of these drugs is relatively specific, restricted to certain types of cell. The response in many cases is elicited so rapidly that it is almost certain that it is the membrane itself which is the receptor system, for insufficient time elapses after application for a significant amount of the drug to diffuse into the interior of the cells. So there is little doubt that the surfaces of certain types of cells must be specifically adapted to certain drugs. At present we cannot say of what that adaptation consists. But it is probable that consideration of the structural peculiarities of different drugs will give us a clue. Consider adrenaline: like the great majority of substances which act on the cell surface, it is a base and consequently, as the cell surface is negatively charged, it will tend, for purely electrostatic reasons, to be in higher concentration at the cell surface than elsewhere. Secondly, it is partly hydrocarbon in character; this again will cause it to accumulate at the oil-water interface of the cell membrane. Thirdly, it has three hydroxyl groups in close proximity to an aromatic (benzene) ring; the investigations of Schulman and Rideal suggest that this will enable the molecule to interact with the monolayer of protein adsorbed on the surface of the cell membrane; this is a third factor increasing the relative concentration of adrenaline at the cell surface. Now any one of these factors taken alone would be insufficient to cause a large increase in adrenaline concentration at a cell surface, but all three factors operating simultaneously may have a remarkably large effect. For drugs of the type we have been discussing it is possible to calculate that, with all three factors operating simultaneously, the concentration at the surface of a cell may rise to between 10^2 and 10^8 times that in the bathing fluid. For example, it is theoretically possible that when a cell is bathed by a solution containing 1 in 10^7 adrenalin _{α} the concentration at the surface of the cell is of the order of 1 in 100. Thus, by consideration of these three mechanisms of adsorption on the cell surface, we find that drugs, present in bulk in minute amounts, may at a suitable surface be present in very substantial amounts. Turning to consider the specificity of the reaction of the cell surface to certain drugs, we are at once confronted with the possibility that the specificity may reside in the steric arrangements at the cell surface, for only if the molecular pattern of the cell surface is exactly correct, and presents its charged groups, oil surface, and protein grouping, all in a correct spatial arrangement, will all three mechanisms of adsorption be able to act

simultaneously. In terms of such a conception it is easy to see that even minor alterations in molecular structure, such as addition of CH_2 groups, or displacement of NH_2 groups along a hydrocarbon chain, will profoundly affect the possibility of specific adsorption at the cell surface.

When we turn to the result of adsorption of drugs on the cell surface, the first thing to consider is the effect such adsorption will have on the permeability of the cell membrane to potassium and on the magnitude of the resting potential, for the excitability of cells appears, in the vast majority of cases, to be irrevocably bound up with this potential. We know from the work of many investigators, such as Höber, Netter, Osterhout, and Cowan¹⁵, that when there is no potassium gradient across the cell membrane there is no resting potential, excitability is lost, and in at least one case (plant cells, Blinks³) the permeability of the cell to ions is greatly increased. Solandt has shown that under these conditions the heat production of muscle is many times greater than under normal conditions. It is evident that the cell surface is a very labile system, and that if adsorbed drugs are able to produce even minor changes in the packing and orientation of the membrane molecules, the excitability of the cell will be profoundly affected.

Similarly, the changes in protoplasmic viscosity following stimulation of cells by electric currents or ultraviolet light, or by changing the ionic environment (Heilbrunn and Daugherty⁵⁵), are probably closely connected in most cases with the condition of the cell membrane. Potassium solutions, for example, cause liquefaction of the cortical gel layer of *Amoeba dubia*, whereas calcium solutions increase the rigidity of the gel layer. From what we know of the actions of potassium and calcium on the resting potentials of other cells, it is quite possible that liquefaction of the cortical gel is due to discharge of the resting potential, and increased rigidity to increase of the resting potential.

iii. FERTILIZATION, ARTIFICIAL PARTHENOGENESIS, CELL DIVISION, AND PROTOPLASMIC STREAMING

In the process and results of fertilization some of the various functions of the cell surface, the cell membrane, and the ectoplasm appear particularly clearly. In the first place, in many cases (sea-urchins and *Nereis*) attachment of the sperm involves an agglutination-type interaction between the sperm surface and the egg surface, due to the presence of a substance on the egg surface which F. R. Lillie has called fertilizin. Fertilizin is soluble in water and can readily be washed out of fresh ripe unfertilized eggs. A drop of fertilizin solution will cause agglutination of a sperm suspension. After repeated washing sea-urchin eggs may no longer be fertilizable, and fertilizin can then no longer be detected in the final wash-waters. On the other hand, attempts to show the existence of fertilizin in certain other forms have failed, so that it is doubtful

whether this agglutination-type reaction is of major importance. But there is no doubt that it does facilitate fertilization of sea-urchin eggs.

The details of the fertilization process differ considerably from species to species. As an example we shall consider *Arbacia*, partly because it is one of the few forms in which I have had the opportunity of following the details of fertilization with care, partly because it is a common form concerning the behaviour of which there is comparatively little controversy. A short time after insemination with a few spermatozoa, one or more is firmly attached to the cell surface. As the spermatozoon comes close to the plasma membrane, streaming movements in the cell surface may become more pronounced. The tip of the spermatozoon enters the surface and a cloudy appearance sweeps over the surface from the site of sperm attachment. Just states that the cloudiness is in the ectoplasmic layer, but it may equally well be on the surface of the plasma membrane: sudden compression of a protein monolayer produces a very comparable cloudiness. The cloudiness clears up, beginning at the site of sperm entry, in a second wave, and a cone forms on the egg surface tending to engulf the sperm head. Suddenly the sperm head passes inside the cone and the fertilization membrane begins to lift away from the surface in the vicinity of the cone. From this point a wave of membrane separation sweeps round the cell and then for a period of about half a minute the space between the fertilization membrane and the plasma membrane increases. The fertilization membrane arises from the vitelline membrane, and if the vitelline membrane is removed prior to insemination sperm entry may still occur, but no fertilization membrane appears. Loeb showed that if proteins are added to the sea-water in which insemination occurs the fertilization membrane is not lifted, which strongly suggests that it is elevated by the colloid osmotic pressure of proteins lying between the fertilization and plasma membranes. Chambers suggests that filaments extend between the two membranes: these filaments can readily be torn with a micro-needle.

Prior to the visible elevation of the fertilization membrane, a wave sweeps round the cell from the site of sperm entry, which in some way inhibits the entry of additional spermatozoa. The additional sperm are then usually lifted off by the rising fertilization membrane, but Driesch has shown that removal of the fertilization membrane does not enable additional sperm to enter. Evidently sperm entry induces a fundamental change in the relationship between spermatozoa and the cell surface. Many attempts have been made to show that this is paralleled by permanent physical changes in the cell surface, but so far no great success has attended these attempts: changes in permeability and electrical capacity, for example, are found in some species but not in others. An interesting transient change has been found by Just. If placed in tap-

water before insemination, cytolysis of *Nereis limbata* eggs occurs in 3 or 4 minutes. But immediately after the sperm entry, cytolysis occurs in about 25 seconds. This increased susceptibility disappears fairly quickly. A similar increased susceptibility also appears in the eggs of *Arbacia* and of *Echinarachnius*. During this cytolysis the region of greater susceptibility appears to be that from which the fertilization membrane has lifted, as with an egg in which partial elevation of the membrane has occurred, the outflow of cytoplasm never proceeds from a region from which the membrane is not lifted. It remains to be seen whether this increase in susceptibility to cytolysis is a common feature in many species. Polyspermy only occurs with large eggs (with which, as Just remarks, we may suspect that the entry of more than one sperm is due to the long time required for the cortical reaction to spread round the cell), or with small eggs which are stale, or which have been treated with some reagent such as nicotine, high temperature, or acid. Cross-fertilization between different species takes place with varying ease, usually with much less ease than specific fertilization, and is frequently assisted by abnormal treatment of the egg. These observations show us that fertilization is in part specific, in part non-specific, and that the relationship between spermatozoon and egg cell which enables fertilization to occur is not a function of one variable only. Gray pointed out that immunological or chemical views of the fertilization process, such as those of F. R. Lillie^{65,66}, meet grave difficulties in explaining the non-specific aspects of fertilization, such as cross-fertilization. The view of R. S. Lillie, that the initial action of the spermatozoon is to initiate a change in permeability of the plasma membrane, is capable of explaining the non-specific aspects, but not, unless modified, of explaining the specific aspects of the phenomenon. So far, then, as it is possible to see at the moment, activation by a spermatozoon involves an apparently non-specific trigger mechanism, the operation of which is facilitated if spermatozoon and egg have a specific relationship, which is probably a function of the spatial or chemical organization of their surfaces.

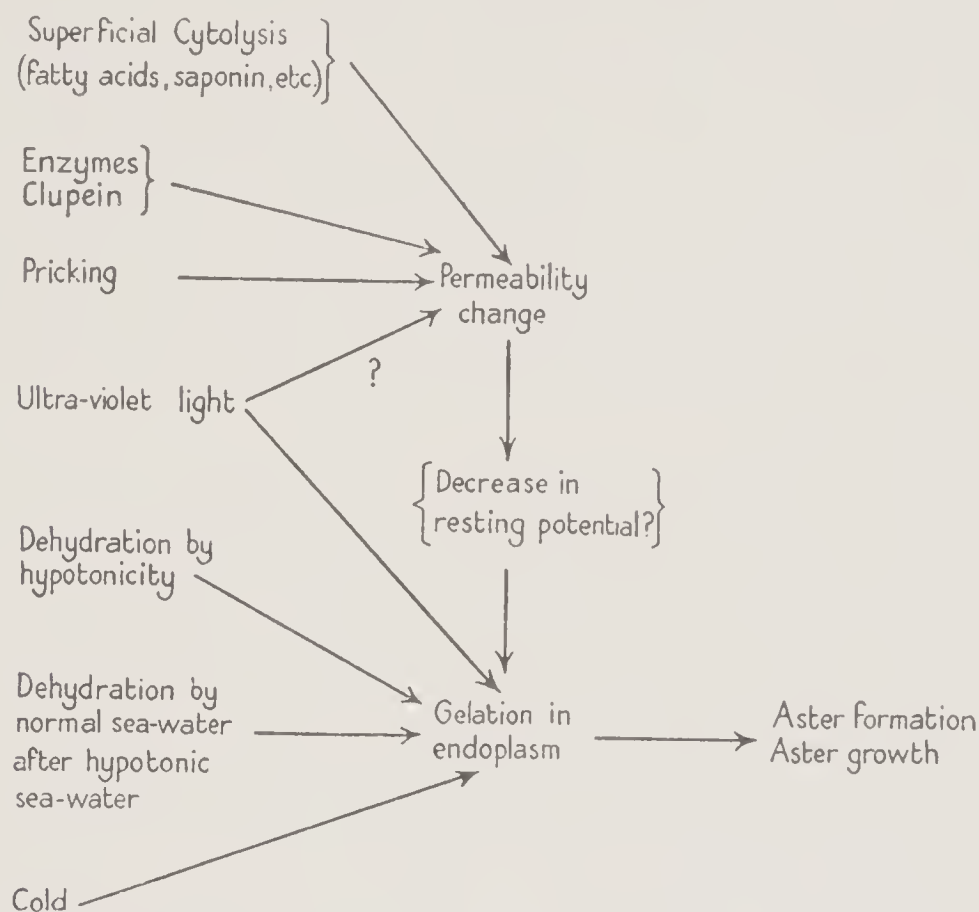
The permeability changes suggest that there is a profound change in the cell surface accompanying fertilization. The school of Runnström has made a number of particularly important observations bearing on this point. Thus, the scattering of light by the cortex changes profoundly on fertilization, and Monroy and Montalenti have shown that there is a change in the birefringence of the cortex. The birefringence undergoes a cyclical change which is correlated with aster growth and with the appearance of the egg surface during cytolysis. Moser and Runnström have studied granules in the cortex of the egg. On fertilization these tend to move to the surface of the cell, and eventually become incorporated in the fertilization membrane. It remains to be seen which of these changes are of general importance in relation to fertilization, and

which are of interest only in the fertilization of one or more species of sea-urchin.

Artificial parthenogenesis may be produced by a great variety of treatments of the egg. It is an effect dependent only on the intactness of the cytoplasm, for E. B. Harvey⁴⁷ has recently shown that enucleated halves of *Arbacia* eggs when treated by parthenogenetic reagents may form fertilization membranes and divide, reaching a blastomere of some 500 cells before serious abnormalities in development appear. Parthenogenetic reagents may be divided roughly into two groups, though some substances probably fall into both groups. The first group consists of cytolytic agents, such as soap and saponin, whose action is almost exclusively on the cell membrane. The second group consists of treatments or reagents whose action is primarily on the bulk of the cytoplasm, such as hypertonicity and cold. Development may occur without elevation of the fertilization membrane. The action of all these reagents is to cause the production of asters in the cytoplasm: many workers, e.g. Morgan, Wilson, Conklin, and Herlant, have shown that frequently very many small asters are produced by these reagents. Some workers, e.g. Loeb^{68,69}, have concentrated their attention on the action of the various reagents on the cell surface; others, e.g. Just, have concentrated mainly on the effects on the bulk of the cytoplasm, and regard dehydration as the essential step in activation. Both of these extreme views seem improbable: it is most unlikely that cold and moderate hypertonicity can be regarded as cytolytic agents. And, on the other hand, ultraviolet light, pricking, or small concentrations of fatty acids, all of which are effective parthenogenetic agents, can hardly be regarded as dehydrating agents. A more probable view is that the essential function of parthenogenetic agents (if indeed there is only one essential function) is to promote aster formation.* Asters are gels, probably of myosin-type proteins. Their formation in the cytoplasm can therefore probably be promoted (*a*) by direct action on the cytoplasm, and (*b*) by stimulation of the cell surface. This gives us the scheme of Text-fig. 5. Cytolytic agents and pricking must give rise to a change in permeability and this, possibly as a result of decreasing the resting potential, may cause an increase in the probability of gelation in the endoplasm. The process is envisaged as being closely analogous to the increase in endoplasmic viscosity in *Amoeba* following treatment with K⁺ (Heilbrunn and Daugherty⁵⁵). Changes in tonicity all involve at some stage a dehydration process: this will favour gelation, as also will low tempera-

* The present author does not wish to discuss whether division in parthenogenesis is due mainly or in part to the formation of fresh asters, or whether it is wholly or in part due to the growth and division of the female aster, but simply to emphasize that conditions favouring gelation will promote growth and formation both of new and of pre-existing asters.

tures, without the intervention of a change in permeability. Where excessive changes in tonicity are used, superficial cytolysis may also be involved. Ultraviolet light may act in both ways, as Tchakotine⁹⁷ has shown that ultraviolet radiation has a direct action on the cell membrane as well as on the cytoplasm. Whether this proposed scheme is correct or not, it is certain that the only visible initial common feature of the



TEXT-FIG. 5. Diagram illustrating how various types of parthenogenetic reagents may all cause aster growth or aster formation.

action of the various parthenogenetic reagents is the initiation of gelation and aster-formation. In view of the importance of sol $\rightleftharpoons$ gel changes and amphiaser formation in mitosis and cell division, it seems likely that working backward from this common attribute of the various reagents is more likely to give a clue as to the nature of the mechanism involved than is consideration of the properties of any small group of reagents.

There is no evidence that the cell surface is particularly involved in nuclear division. In cytoplasmic division the ectoplasm at least plays a large part. Originally, attention was focused more on the lipoid layer of the cell membrane, and changes of surface tension were held by many to be responsible for cell division, but for reasons already discussed it is now certain that surface tension plays at most a very minor role in normal cell division. Recently more attention has been paid to the ectoplasmic or cortical gel layer. Chambers has shown that egg fragments having cortical gel may be fertilized and will divide, whereas fragments

lacking cortical gel will not divide. Subsequently, further micro-dissection studies have shown that the impingement of the cleavage furrow is due to an invagination of the cortical gel layer: the process is accompanied by increased gel formation, and as the gel invaginates the surface membrane adheres to it and follows the course of the impinging furrow. When the invaginating gel girdle meets in the centre, fusion takes place, together with retraction of gel from the small tube still uniting the blastomeres, until finally only a very narrow thread of cytoplasm remains to unite the two cells. This activity of the cortical gel may be inhibited at any time by the application of sufficient hydrostatic pressure to cause liquefaction of the gel (Marsland and Brown; see Chapter II). The action of pressure is reversible in many ways. The protoplasmic thread uniting the blastomeres may persist, possibly indefinitely in some cases, and in others until movements or surface-tension forces break the thread. How far similar changes are involved in division of plant cells is unknown.

Many workers have remarked upon the streaming movements of the cytoplasm in dividing cells. Chambers has recently remarked that in cytoplasmic division much of the streaming is closely associated with gel formation in the cortex. It is therefore most interesting that Marsland has found that streaming in *Elodea* cells is associated with gelation, and that streaming is abolished by application of sufficient hydrostatic pressure to liquefy the cortical plasmagel. Marsland suggests that the streaming process is due to a wave of gelation and dissolution of gel passing round the cell and that, for example, the fluid is pushed round by the advancing wave of gelation, just as fluid may be pushed along a rubber tube by causing a constriction to pass along the tube. The recent growth of evidence that $\text{sol} \rightleftharpoons \text{gel}$ changes are an essential part of the apparatus of protoplasmic movement is of the greatest importance. Those interested in other potentialities of the plasmagel may consult Needham's book, *Order and Life*⁸².

From the foregoing account it will be realized that rapid and dramatic changes are occurring in our conception of the cell membrane and its relationship with the rest of the cell. The fundamental principles underlying this development, however, are those which were introduced by such pioneers as Ringer, Overton⁸⁴, Bayliss², Loeb^{68,69}, Morgan^{77,78}, R. S. Höber and F. R. Lillie^{65,66}, and Hardy. Perhaps it is not too much to hope that the coming decade will see connexions established between this field and the enzymatic processes which furnish the supply of energy for cellular activity. Now that we are approaching the problem of molecular organization in protoplasm, it is reasonable to suppose that we may also make contact with the biochemical processes which, as Hopkins has so lucidly explained, must also be dependent on a complex molecular organization.

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CHAPTER V

NUCLEUS, CHROMOSOMES, AND GENES

By M. J. D. WHITE

i. INTRODUCTION

SINCE the publication of the first edition of this book a great deal has been learned about the chemical structure of the chromosomes (mainly by Mirsky and Pollister, following on the pioneer work of the Stedmans^{201,202}, and about the biochemical nature of gene-action (by Beadle, Tatum, and their associates).

On the one hand, it now appears that the chromosomes consist, in the main, of four types of chemical compounds, namely, two kinds of nucleic acids, in one of which the carbohydrate part of the molecule is *d*-ribose, while in the other it is desoxyribose; together with two kinds of proteins, one of which is a histone or a protamine, while the other is a tryptophane-containing protein of higher molecular weight than the first.

On the other hand, it has been shown by the second group of workers (Beadle^{18,19,20,21,22}; Tatum, Bonner, and Beadle²⁰⁹; Beadle and Tatum²⁵; Beadle, Mitchell, and Nyc²⁴; Srb and Horowitz²⁰⁰) that each biochemical reaction in the fungus *Neurospora* (such as the conversion of citrulline to ornithine or that of anthranilic acid to indole) is determined genetically, and that the more complex biochemical conversions (such as that of kynurenine to nicotinic acid) are determined by several genes, each one of which is responsible for a particular step in the conversion. Thus the formation of citrulline from ornithine involves the addition of both $\begin{array}{c} | \\ \text{CO} \\ | \end{array}$ and $\begin{array}{c} | \\ \text{NH} \\ | \end{array}$ groups, and apparently each of these steps is controlled by a separate gene. When any one of these genes mutates to an 'amorph' (i.e. a totally inactive state) the particular reaction which it normally controls no longer occurs, and hence the precursor-substance accumulates in the tissues of the organism, is excreted in abnormal quantities, or is used up in an alternative biochemical reaction. The behaviour of genes is hence very like that of enzymes (as had long been suspected on general grounds), and it seems likely that each gene is a precursor or producer of a specific enzyme which determines a particular biochemical reaction in the cell.

These two lines of attack on the central problem of the nature of the gene are thus approaching one another, but they have not yet met. In other words, although we have some idea of the gross chemical composition of chromosomes, we do not know anything certain as yet about

the composition of the individual genes of which they are made up, so that we are still totally ignorant as to the nature of the chemical differences between one gene and another and, in particular, of the differences between allelic genes which have arisen from one another by mutation. Nor do we know whether the chromosomes are entirely composed of genic material, or whether, as seems more probable, they also contain materials (either inter-genic substance between the genes or 'matrix' surrounding them) which do not form part of the genes themselves.

The researches of the past few years and, in particular, the work of Sonneborn^{195,196}, have tended to focus attention on the self-duplicating particles of the cytoplasm. But this in no way detracts from the importance of the chromosomes as the main self-duplicating organs of the cell. Thus an analysis of the molecular structure of the genes and chromosomes is clearly one of the central problems of biology at the present time: it is a prerequisite to any real understanding of the process of mutation, and consequently of the physical basis of variation and evolution. In order to evaluate all the more recent work, however, it is necessary to have some understanding of the basic concepts which have been developed by classical cytological methods.

ii. THE NUCLEUS

Nuclei are masses of relatively fluid material ('nuclear sap') surrounded by a membrane and containing the chromosomes. We still know very little about the nature of the nuclear membrane, and especially about its permeability to molecules of different kinds, although this is really one of the central problems of cell physiology.

Most nuclei are spherical, although ovoid ones are not uncommon in tall, columnar cells. In some cases, where the nuclei are complex ramifying structures—as they are in the spinning-glands of some insects (Vorhies²¹⁵), and in the salivary glands of waterbugs (Geitler⁷⁰)—the cytoplasm contains large quantities of secreted material, in many cases the silk protein, *sericin*. Here the structure of the cytoplasm may be responsible for the nucleus assuming this peculiar arborescent form, which becomes more complex as the cell grows to maturity. This can hardly apply in two other classical cases of irregularly shaped nuclei, those of the polymorphs of vertebrate blood, and the macronuclei of some ciliates, such as *Stentor*, *Condyllostoma*, and *Spirostomum ambiguum*.

We shall see later that all these irregularly shaped nuclei are probably highly polyploid, containing hundreds or thousands, and in some instances tens of thousands of chromosomes. It is noteworthy that the salivary gland cells of most Dipterous larvae, some of which secrete large quantities of silk, are strictly spherical. In an irregularly shaped

nucleus the surface area of the nuclear membrane is, of course, greater, relative to the volume, than in the case of a spherical nucleus, and this may have a functional significance in facilitating nucleo-cytoplasmic exchanges. The only known case of an irregularly shaped salivary gland nucleus in the Diptera is in the single 'super-giant' cell at the base of each salivary gland in the midge *Lestodiplosis* (White²²²).

It is probable that in the future all the various types of resting nuclei will be found to have significance in relation to the particular functions of their cells. The diversity of nuclear structure is clearly related to histological differentiation, and when we know more about the role of the nucleus in the life of the cell we shall doubtless understand the different morphological types of nuclei better. The statement that: 'Throughout plants and animals the cell nucleus has a uniformity of structure corresponding to the uniformity of its work' (Darlington⁵³) is only a crude first approximation to the truth.

iii. THE CHROMOSOMES

a. The Structure of the Chromosomes as Revealed by Microscopy

Each chromosome is composed of a linear sequence of parts, having different properties (genetical, chemical, &c.). All 'normal' chromosomes have two ends, but it is possible, by means of irradiation with X-rays, to break off small pieces from both ends; in this case the two ends of the central portion which is left may join up so as to produce a ring-chromosome which has 'no beginning and no end'. The properties of such artificially produced ring-chromosomes have been studied in *Drosophila* (Morgan¹⁴³; Catcheside and Lea⁴⁶; Bauer¹⁶) and in the maize plant (McClintock^{118,120}): they are liable to undergo various kinds of 'accidents' at mitosis, so that it is hardly surprising that they have never been encountered in any wild species of animal or plant.

All 'ordinary' chromosomes possess one special region, the *centromere* (*kinetochore* or *spindle-attachment* of some authors), which plays a special role during mitosis, since it connects the rest of the chromosome to the mass of gelated material, mainly derived from the nuclear sap, which forms the spindle. The centromere, like other parts of the chromosome, possesses the power of self-duplication, which is followed by 'splitting', although there is a good deal of evidence that it does not split at the same time as the rest of the chromosome thread, and it may also undergo duplication at a different part of the mitotic cycle.

Under the microscope the centromere region usually appears as a non-staining gap in the substance of the chromosome: in all probability it is a region containing little or no nucleic acid. In some instances a minute dark-staining granule has been detected in the middle of the centromere region, and some authors have assumed that this is the true

centromere; but in most cases such a granule cannot be seen. Trankowsky²¹⁴ and Iwata⁹³, working on plant material, and Schrader¹⁷⁷, who worked on the Urodele, *Amphiuma*, are in agreement that each meiotic bivalent possesses four of these 'attachment chromomeres' in the first meiotic division, i.e. one for each chromatid.

Chromosomes with strictly terminal centromeres apparently do not exist in nature (White²¹⁷; Coleman⁴⁷). When such a chromosome is produced artificially by breaking a normal chromosome in the immediate neighbourhood of the centromere, or actually through the centromere, it is either eliminated in the course of a few divisions or converts itself into an 'isochromosome' with two genetically identical arms and a median centromere (Rhoades¹⁶⁹).

It is possible, by means of irradiation with X-rays, to obtain chromosomes with several centromeres. Such chromosomes arise when two or more chromosomes of the original set are broken transversely and the portions rejoin in such a manner that a compound chromosome containing parts of several of the original ones is formed. In most organisms such 'dicentric', 'tricentric', &c., chromosomes are unable to function efficiently at mitosis, since it frequently happens that the several centromeres of a single daughter-chromosome do not pass to the same pole of the spindle at anaphase. It was originally shown by Mather and Stone¹²⁸, and has frequently been confirmed since, that when two centromeres situated in the same chromosome pass to opposite poles of the spindle at anaphase the region between them will be stretched in the spindle and will either (1) prevent the two groups of chromosomes from separating, so that they become included in a single nucleus, or (2) be unable to pass to either pole, so that it fails to get included in either daughter-nucleus, or (3) the stretched region will simply break under the tension imposed on it by the elongating spindle. It was shown at the same time that 'acentric' fragments, which lack a centromere altogether, are likewise unable to function properly at mitosis, since they never become attached to the spindle at all.

There is, however, one organism in which it is definitely known that the chromosomes possess many centromeres. This is the Nematode worm, *Ascaris megalocephala*, where it has long been known that the 2 or 4 chromosomes of the germ-line (2 in the variety *univalens*, 4 in the variety *bivalens*) break up spontaneously during certain of the cleavage divisions into a number of much smaller portions, but only in those nuclei which are destined to pass into the somatic tissues of the worm. Here it is clear from the results of irradiation experiments that each segment possesses its own centromere, which is functional even before fragmentation, and that the germ-line chromosomes are accordingly *polycentric*. Why the centromeres in one daughter-chromosome always pass to the same pole at anaphase in the germ-line cells is

not entirely clear—perhaps they are too close together to be able to pass to opposite poles.

In certain orders of insects, such as the Hemiptera, no centromeres have ever been seen under the microscope, and at mitosis each chromosome appears to be attached to the spindle along its entire length (instead of at one point only). It is uncertain whether such chromosomes have a 'diffuse-centromere activity' (Schrader^{178,180}) or a large number of small centromeres scattered at intervals along their length, as appears to be the case in *Ascaris*. In these bugs it appears from the work of Hughes Schrader and Ris⁹¹ (on the scale-insect *Steatococcus*) that if the chromosomes are broken up into a number of smaller portions by irradiation with X-rays, each of the fragments is able to function properly in subsequent mitoses, all portions becoming attached to the spindle, as if they possessed centromeres or a 'diffuse-centromere activity'.

During the early prophase stages of the first meiotic division the chromatids resemble a string of beads, numerous small, dark-staining granules being connected together by a faintly staining thread. These granules have received the name of chromomeres. Some workers have claimed that they are artefacts or optical illusions based on misinterpretation of a spiral chromonema. It has been pointed out by Kaufmann⁹⁸, however, that it is hard to reconcile these views with the fact that individual chromomeres are frequently identifiable from one cell to another (Heilborn⁷⁸; Huskins and Smith⁸⁷; Longley¹¹⁵). Moreover, it is clear that the chromomeres are Feulgen-positive (i.e. they contain desoxyribose nucleic acid) while the portions of the chromonema between them are Feulgen-negative. It is thus difficult to accept the view that the chromomeres and interchromomeric connexions only differ in degree or mode of coiling.

The total number of chromomeres in the chromosome set of a species can only be stated in the form of a lower limit, since it is clear that the larger chromomeres in most fixed and stained preparations are compound, i.e. they consist of several smaller chromomeres fused together. In the plants *Lilium* and *Aloë*, Belling²⁶ estimated the number of 'ultimate' chromomeres at 2,500 and 1,400, although even here it is unlikely that all of them were detected, since the smallest ones are only just resolvable with the microscope.

Muller¹⁴⁵ has used the term telomere for the 'natural' chromosome ends to distinguish them from 'artificial' ends, which are produced when a chromosome is broken as a result of irradiation or by other means. Some authors have used the term to indicate the terminal members of the gene-string, while others have suggested that it should be applied to some kind of hypothetical 'pellicle' over the ends of the chromosome. Morphologically the term lacks precision, but it has been used to emphasize the important principle that when structural rearrangements

occur (see Section *h*) the 'telomeres' are incapable of assuming an interstitial position in the chromosome, and no interstitial portion of the chromosome is capable of assuming a terminal position, i.e. of functioning as a telomere. Some supposedly 'terminal' inversions of chromosome regions have been described in wild populations, but it is not quite certain that they were strictly 'terminal', i.e. that there was not a minute region beyond the limits of the inverted segment. The telomere concept obviously requires some modification in the case of *Ascaris megalocephala*, where the ends of the germ-line chromosomes are cast off in the somatic cells, the central portions undergoing fragmentation into a large number of much smaller chromosomes.

Nucleoli, which were formerly regarded as independent cell organs, are now known to be simply lateral, bladder-like expansions of special chromosome regions. Usually the place where a nucleolus arises is marked by a 'constriction' which in most cases lies in a heterochromatic segment. Many diploid species of animals and plants have a single pair of nucleolar chromosomes, in which case the nucleoli of the two homologues may be fused together. There are, however, a number of undoubtedly diploid species which have more than one pair of nucleolar chromosomes (Bauer¹⁶; White²²⁶). Usually the nucleoli disappear before metaphase; they may be absorbed into the substance of the chromosome.

Most authors have tended to regard the nucleoli as temporary storage-reservoirs of material, which is transferred to the chromosomes shortly before metaphase. But they are probably significant in other ways as well. Occasionally nucleoli have been observed to 'bud-off' smaller masses of nucleolar material which disperse throughout the nuclear sap (in snail oocytes, according to Serra and Queiroz Lopes¹⁸⁹). This nucleolar material is almost certainly passed into the cytoplasm; Duryee⁶³ has claimed that in amphibian oocytes the small nucleolar masses actually pass through the nuclear membrane, but Painter and Taylor¹⁵⁹ could find no evidence of this.

Apart from the centromere and, in some cases, the nucleolus-organizing region, many chromosomes possess so-called 'secondary constrictions' (the term was coined to distinguish them from the so-called 'primary constriction', which marks the position of the centromere).

Secondary constrictions appear under the microscope as non-staining gaps in the substance of the chromosome. Thus, in the second chromosome of *Drosophila melanogaster* the 'left' limb is divided by a secondary constriction into two portions, which are sometimes so widely separated that they look almost like different chromosomes (Kaufmann^{96,98}).

It has been suggested that the secondary constrictions represent the positions of heterochromatic regions, and a comparison has been made with certain 'differential regions' of plant chromosomes which only stain

faintly after the organism has been exposed to low temperatures (Darlington and La Cour⁵⁶). A more probable interpretation would seem to be that they simply represent unusually long inter-chromomere regions.

If electron microscopy could be used to reveal the internal structure of chromosomes we should, no doubt, learn a great deal about the size and number of chromomeres, the number of chromonemata at various stages of mitosis, and many other matters bearing on the number and size of the genes, their methods of reproduction, &c. Unfortunately the electron-microscope studies of chromosomes which have been carried out up to the present time (Buchholtz³⁵) merely indicate that the technical difficulties are still too great for any really significant results to be obtained. If, at some future time, the technical difficulties are overcome, a whole new era of cytology will be opened up; but at present the outlook in this direction is not encouraging, although electron microscopy has been used with considerable success on bacteria and virus-particles.

Usually the chromosomes seem to be distributed at random inside the nuclear membrane during prophase, but some exceptions are known. Thus, heteropycnotic chromosomes (see Section c) very frequently lie against the inside of the nuclear membrane rather than in the middle of the nuclear cavity. At metaphase it is usual for the larger elements of the chromosome set to lie round the periphery of the 'plate', the smaller chromosomes lying in the centre, but in some species the arrangement is less orderly, and in others there is a 'hollow spindle' (i.e. no chromosomes occupy the central portion of the plate). As a rule there is no tendency for homologous chromosomes to pair or to lie particularly close to one another in ordinary somatic divisions; but in the two-winged flies (Diptera) homologous chromosomes may be rather closely paired during the prophase stages, and even at metaphase they tend to lie together on the plate, parallel to one another. This phenomenon of 'somatic pairing' is especially characteristic of the Diptera, and claims to have established its existence in other groups of organisms are not very convincing.

b. The Chemical Composition of Chromosomes

According to the latest researches of Mirsky, Pollister, and Ris (Mirsky¹³⁷; Mirsky and Ris^{141,142}; Mirsky and Pollister^{138,139,140}; Pollister and Ris¹⁶²; Ris¹⁷⁰) there are four main types of chemical compounds present in chromosomes: ribose nucleic acid (RNA); desoxyribose nucleic acid (DNA); proteins of a histone or protamine type; and a tryptophane-containing protein.

The nucleic acids are polymers of nucleotides, each nucleotide being composed of one molecule of phosphoric acid, a pentose sugar, and a purine or pyrimidine group. The various kinds of nucleotides have different purines or pyrimidines in their molecules. Apart from this,

there are two main types of nucleotides, which differ in the nature of the pentose. In those which form RNA the sugar is *d*-ribose, while in those which form DNA it is desoxyribose.

Four different nucleotides have been identified in RNA (the adenine-, guanine-, cytosine-, and uracil-nucleotides), and four in DNA (the adenine-, guanine-, cytosine- and thymine-nucleotides). It is not certain, however, that these are the only units which enter into the composition of the nucleic acids, nor is it certain that they exist in equimolecular proportions in the polynucleotide chains, as was formerly believed (Gulland⁷⁷).

The nature of the chemical linkages between the successive nucleotides in a nucleic-acid molecule is not fully understood, but the molecular weight of the fully polymerized chain-molecule is known to be very high. Signer, Caspersson, and Hammarsten¹⁹⁰ determined the molecular weight of DNA by the ultracentrifuge and found it to lie between 500,000 and 1,000,000, while Creeth, Gulland, and Jordan (see Gulland⁷⁷), using similar methods, estimated it at 3,000,000. Both groups of workers agree that the molecules are long, thin rods whose length is about 300 times the breadth (Signer, Caspersson, and Hammarsten) or 710 times the breadth (Creeth, Gulland, and Jordan). Since the average molecular weight of a nucleotide is about 330, it seems likely that the polymerized nucleic-acid molecules in the chromosomes consist of 1,500 to 9,000 nucleotides. But it is not known whether the different kinds of nucleotides are arranged in the polymerized molecule in any particular sequence, although this seems probable from the X-ray investigations of Astbury⁴. Obviously, the possibilities of isomerism in the nucleic acids are considerable, and it has been pointed out by Gulland⁷⁷ that their potentialities for specificity are not unlike those of the proteins, so it is not impossible that the specificity of gene-action depends in part on the nucleic-acid component of the gene rather than on the protein part alone, as has usually been assumed in the past. On the other hand, it must be admitted that there is no critical evidence for the existence of many different stereoisomeric nucleic acids.

The early and classical investigations on the chemistry of the nucleus were carried out mainly on the sperm of herring, salmon, and other teleost fishes, on the red cells of bird blood, and on the mammalian thymus (Miescher¹³⁶; Ackermann¹; Steudel²⁰³; Kossel¹⁰⁴). This work suggested that the chromosomes consisted entirely of histone- or protamine-nucleate, that is, of a compound between a nucleic acid and a basic protein of low molecular weight. According to Stedman and Stedman²⁰², in the case of salmon and herring sperm-heads this protein is a protamine (i.e. a relatively small molecule in which arginine accounts for about 90 per cent. of the total N and which contains no tyrosine or tryptophane), while in cod sperm-heads it is a histone (a somewhat

larger molecule in which arginine only accounts for about 25 per cent. of the total N and which contains some tyrosine but little or no tryptophane).

The physiological significance of this difference between the sperm-nuclei of different teleost fishes is obscure, but in the salmon, where the sperm-heads contain protamine, the spermatogonia and spermatocytes of immature testes contain histone (Miescher¹³⁶; Stedman and Stedman²⁰²). It would appear, therefore, that during the later stages of spermatogenesis the histone of the chromosomes is converted into or replaced by protamine. The somatic nuclei of the salmon (liver and erythrocytes) contain histone, but not protamine.

Thus far the work of the Stedmans confirmed the work of Miescher, carried out nearly two generations ago. But in addition to the protamine or histone they also found another protein in the material they studied. This was insoluble in dilute acids and alkalis, gave strong tryptophane, tyrosine, and arginine reactions, and was given the name *chromosomin* by the Stedmans.

Using entirely different methods, Mirsky and his collaborators have arrived at somewhat similar results. Mirsky first grinds the material to be studied (thymus, liver, fish-sperm, &c.) in a Waring blender or a colloid mill at a temperature of 3–4° C. The finely ground material is then strained through fine cloth and subjected to differential centrifugation in order to obtain isolated chromosomes, free from other cellular material. The isolated chromosomes are kept as a suspension in 0.14 M NaCl at a pH of approximately 6.3. When such chromosomes are placed in M NaCl about 90 per cent. of their weight passes into a viscous opalescent solution. Chemical tests show that this soluble fraction consists almost entirely of nucleohistone. The rest of the chromosomal material (approximately 10 per cent. by weight) remains in the form of a 'residual chromosome', a sort of ghost of the original body, but which still shows a typical chromosomal structure, with differentiation into euchromatic and heterochromatic regions.

These residual chromosomes also consist of nucleoprotein materials. The nucleic acid is mainly RNA, there being about four parts of this to one part of DNA in the case of residual chromosomes obtained from thymus lymphocytes. The protein part of the residual chromosome is the tryptophane-containing protein (chromosomin).

A convenient staining method which has been used by Brachet³¹ enables one to distinguish between ribonucleoproteins and desoxyribonucleoproteins: a mixture of methyl green and pyronin stains the former red and the latter green. In the case of isolated thymus chromosomes, the whole chromosome is stained a bluish green, the soluble nucleohistone green, and the residual chromosomes red (Mirsky¹³⁷).

No doubt a great deal more will be learned about the chemical

structure of chromosomes when adequately purified enzyme preparations (ribonuclease and desoxyribonuclease) are generally available. Until recently most of the preparations of these enzymes which have been used in experiments on chromosomes were contaminated, and possessed considerable proteolytic activity.

It has been generally believed that the Feulgen reaction (staining with leuco-basic fuchsin after hydrolysis) is specific for desoxyribose nucleic acid and that the presence of a red coloration after Feulgen staining indicates the presence of DNA in that particular part of the cell, the preliminary hydrolysis with HCl having liberated aldehyde groups from the DNA. The Stedmans have claimed, however, that even though this is so, the compound formed between the stain and the aldehyde groups is fully soluble, so that it would be washed out of the preparation *if it were not for the fact that it is adsorbed by the chromosomin*. Thus, according to them, the red colour in the preparation (a) indicates the presence of DNA, but (b) is retained only in those parts of the cell where chromosomin is present. This interpretation is, however, in conflict with Mirsky's observations on the fractionation of isolated chromosomes with molar NaCl, in which it was shown that the soluble fraction (nucleo-histone) stains with the Feulgen reagent, while the 'residual chromosome' which contains the tryptophane-protein (= chromosomin) only does so very faintly—in agreement with the estimate that it contains four times as much RNA as DNA.

An entirely different method of studying the nucleic acids of the cell was developed by Caspersson, Hammarsten, and Schultz. These workers have studied cytological preparations by ultraviolet absorption spectroscopy; the method depends on the fact that both DNA and RNA give a specific absorption at about 2,600 Å. This absorption depends on the pyrimidine or purine part of the molecule and hence gives no information as to whether ribose or desoxyribose nucleotides are present, nor as to whether they are polymerized or not. Using this method, Caspersson and his collaborators have demonstrated the presence of large quantities of nucleotides, not only in the chromosomes but also in the cytoplasm of all cells which are destined to undergo a series of divisions in rapid succession. Egg cells, meristematic cells in root tips, and imaginal disk cells in insects were all shown to contain cytoplasmic nucleotides, presumably of the ribose type. These cytoplasmic nucleotides are probably the reason why so many embryonic cells have a strongly basophilic cytoplasm. In many types of cells the nucleotides seem to be especially concentrated round the nuclear membrane, so as to give rise in stained preparations to a basophilic 'halo' round the nucleus. On the other hand, the spectroscopic observations mentioned above have shown that the nuclear sap is relatively free from nucleic acids, and so is the spindle which develops from it at mitosis.

'Mature' cells, which are not destined to undergo any further divisions, may or may not contain large quantities of nucleotides in their cytoplasm. Brachet³¹ has shown that cells which produce large quantities of protein always have large amounts of cytoplasmic nucleotides, the largest known quantity being in the silk glands of the silkworm. On the other hand, many types of cells which are physiologically very active (cardiac and striped muscle, lung cells, and those of the endocrine glands) have only small quantities of cytoplasmic nucleotides. The nucleotide content of a cell may be experimentally altered in various ways—thus pancreatic cells show a fall in cytoplasmic nucleotides after intense stimulation (Caspersson, Hydén, and Aquilonius⁴¹).

There is no evidence that the cytoplasmic nucleotides are polymerized to the same extent as those in the chromosomes, and their relationships to the cytoplasmic proteins are also not clear. Since very large quantities of nucleic acids are localized on the chromosomes during cell division, it seems likely that the cytoplasmic nucleotides serve as a source of nucleic acids from which the chromosomes derive their supply. If this is so, the early stages of mitosis probably involve the transference of nucleotides from the cytoplasm to the nucleus, their conversion from ribose into deoxyribose nucleotides (the chemistry of this conversion is not understood), and their polymerization into long chains. Thus we may postulate the existence of a regular cycle of changes, which must be reversed at the end of each mitosis, when the nucleic acid is surrendered by the chromosomes.

It will be realized from the above account that although the main chemical constituents of the chromosomes seem now to have been identified, when we come to consider their relationship to one another and their role in heredity and in protein synthesis we enter the realm of speculation and hypothesis. Such 'elementary particles' as viruses seem to have only one nucleic acid (DNA in the rabies, verruca, and neurovaccinia viruses, RNA in the viruses of polyomyelitis and louping ill, according to Hydén⁹²); but the chromosomes of all higher organisms probably contain both RNA and DNA.

The fact that nucleic acids are present in all types of self-duplicating particles (genes, viruses, plastids, &c.) has suggested that they play an essential role in the synthesis of proteins. But just what this role is is not clear. One suggestion (Muller¹⁴⁸; Spiegelman and Kamen¹⁹⁹) is that the nucleic acids 'funnel energy into the protein synthesizing mechanism'.

An entirely different suggestion as to the relationship between nucleic acids and proteins has been made by Astbury and Bell^{5,6} who have pointed out that the distances between the nucleotides in the long polymerized nucleic-acid molecules is of the same order of magnitude (3.3 Å) as that between the successive amino-acids in a fully extended

polypeptide chain, so as to permit a zipper-like union between them. They have suggested that this apparently accidental correspondence between the spacing of the essential units in these two very different types of molecule may be the essential basis of the auto-reproductive capacity of nucleo-proteins.

On the one hand, we have the suggestion of Gulland that gene-specificity resides in the nucleic-acid part of the molecule, rather than in the protein part, a view which was almost necessary so long as it was believed that the protein was a single protamine or histone, and that histone could be replaced by protamine in the course of spermatogenesis. On the other hand, now that we know of the existence of higher tryptophane-containing proteins in the chromosomes it is evident that they could theoretically, either by themselves or in combination with highly specific nucleic acids, provide the basis for gene-specificity.

Usually the transference of nucleo-protein materials to or from the chromosomes cannot be directly demonstrated by staining methods. In certain cases, however, the chromosomes, when they divide at anaphase (or rather, when their chromatids separate) appear to slough off a certain amount of material, which is almost certainly nucleo-protein, and which then forms a definite body lying in the spindle, mid-way between the two daughter-chromosomes as they pass to the poles. This phenomenon is well shown during the meiotic (maturation) divisions in the eggs of certain moths (Seiler^{182,183}), and a similar process takes place in both the meiotic and the cleavage divisions of the mite *Pediculopsis* (Cooper⁴⁸). In both cases the bodies which are left behind in the equatorial part of the spindle are Feulgen-negative but stain with ordinary basic dyes such as haematoxylin and crystal violet (Plate 1, Figs. 1 and 2). In *Pediculopsis*, where the haploid chromosome number is only 3, it can be seen very clearly that each of the 'elimination-bodies' corresponds to a pair of daughter-chromosomes. The elimination-bodies, both in moths and *Pediculopsis*, are temporary structures, which disappear after each division, being probably broken down by cytoplasmic enzymes. It is interesting to note that in the related mite *Pediculoides* no elimination bodies appear to be formed (Pätau^{160,161}).

Whenever a number of mitoses occur within a short space of time, as during the early cleavage divisions in many embryos, the total number of chromosomes in the body undergoes a very rapid increase. Where do the materials (proteins and nucleotides) needed for the formation of these new chromosomes come from? In all cases they are probably derived from the egg cytoplasm, but in some special cases this probably receives proteins and nucleotides from some external source. Thus it has been suggested by Painter¹⁵⁶ that in insect eggs, where a number of endopolyploid nurse-cell nuclei undergo degeneration at about the time the maturation divisions are taking place in the egg, the consti-

tuent molecules of thousands of chromosomes from these nuclei pass into the egg-cytoplasm, where they are used again in the building up of new chromosomes in the cleavage divisions.

The chemical composition of nucleoli has been considerably debated. Caspersson⁴⁰, using spectrophotometric methods, concluded that nucleoli were rich in histone, and his conclusions were accepted by Darlington⁵³. His methods have, however, been criticized by Pollister and Ris¹⁶² who, using an improved method, found that all the protein in nucleoli of maize-pollen mother-cells was of the non-histone type. They state that these nucleoli are about 3 micra in diameter, have a volume of 1.16×10^{-10} cm.³, and contain about 5×10^{-11} gram of protein.

The nucleic acid present in nucleoli appears to be mainly or entirely RNA. This accounts for the fact that nucleoli do not usually stain with the Feulgen technique. The fact that some nucleoli stain with 'ordinary' nuclear dyes such as carmine, orcein, and crystal violet may be due to differences in the relative amounts of RNA and protein present. It is probable that the chemical composition of nucleoli varies rather widely from one organism to another, so that it would be unwise to regard Pollister and Ris's analysis of the maize nucleolus as necessarily applicable to the nucleoli of other plants and animals.

c. *Heterochromatin and Euchromatin*

Ever since the beginning of the century it has been known that some chromosomes or portions of chromosomes exist in a condensed, dark-staining condition at stages in the nuclear cycle when the remaining chromosomes or chromosomal regions are diffuse and light-staining. This property of *heteropycnosis* is especially characteristic of sex-chromosomes, such as the Y-chromosome of *Drosophila* species and the X-chromosomes of grasshoppers, but is also shown by many autosomal chromosome regions, and sometimes by whole autosomes.

Chromosomal regions which are liable to exhibit the phenomenon of heteropycnosis may be called *heterochromatic* (= composed of heterochromatin), while regions which never show heteropycnosis may be referred to as *euchromatic* (= composed of euchromatin). This terminology is useful, as we frequently have to refer to regions which are not heteropycnotic at the particular stage of the nuclear cycle we are considering, but do show heteropycnosis at some other stage.

Two different types of heteropycnosis are known which seem to be opposite in nature. On the one hand the heterochromatic regions may be thicker and more dark-staining than the euchromatic ones. In this case the heterochromatin has presumably synthesized more nucleic acid, and possibly more protein as well. We shall refer to this as *positive* heteropycnosis. A positively heteropycnotic chromosome in a prophase nucleus looks like a metaphase chromosome.

On the other hand, a heterochromatic region or chromosome may appear thinner and stain more weakly than euchromatic regions in the same nucleus. This type of behaviour may be called *negative* heteropycnosis. Negatively heteropycnotic chromosomes usually have a flocculent, 'fuzzy' outline: at metaphase they still look like prophase chromosomes.

Heteropycnosis may be manifested in resting nuclei, or at any part of the mitotic cycle: it may be described as a 'natural experiment', the euchromatic regions in the same nucleus constituting a standard or 'control' with which the heteropycnotic ones can be compared.

In some organisms only one of the two types of heteropycnosis exists. Thus, in the long-horned grasshoppers (Tettigoniidae) the X-chromosomes are positively heteropycnotic in the resting and prophasic spermatogonial nuclei and also at various stages of meiosis, but neither they nor any of the sometimes extensive heterochromatic regions in the autosomes ever show negative heteropycnosis. In the short-horned grasshoppers (Acrididae), on the other hand, both positive and negative heteropycnosis exists, and the X-chromosome passes through a regular cycle in the course of spermatogenesis, being negatively heteropycnotic during the early spermatogonial divisions (at least in the prophases and metaphases), non-heteropycnotic (i.e. behaving like euchromatin) in the later spermatogonial divisions, and positively heteropycnotic during the prophase of meiosis. In some species, such as *Mecostethus grossus*, they may once more become negatively heteropycnotic in the metaphase of the first meiotic division and then positively heteropycnotic once more in the interkinetic (secondary spermatocyte) and spermatid nuclei. This 'reversal of heteropycnosis' is clearly a fact of considerable interest, but the factors which determine it have not been investigated. Possibly the chemical composition of the protein framework of the chromosome changes in some way, or perhaps the reversal is due to changes in the cytoplasm or in the nuclear sap. It is of interest that in these short-horned grasshoppers the heterochromatic regions of the autosomes, which are quite extensive in many species, do not show this reversal, being non-heteropycnotic in certain stages of spermatogenesis, positively heteropycnotic at other times (Text-fig. 1). If, as we have tacitly assumed, heteropycnosis is a property of the nucleo-protein framework of the chromosome, then there must be at least three different kinds of framework in the chromosome set of these grasshoppers—one in the X, one in the autosomal heterochromatic regions, and a third in the euchromatic regions. More probably there are many varieties of heterochromatin and euchromatin, constituting a continuous 'spectrum', to the two ends of which the terms heterochromatin and euchromatin are somewhat arbitrarily applied.

One property of heterochromatic regions is their tendency to become

aggregated together in resting or prophase nuclei. Thus, in the grasshoppers mentioned above, where there is an autosome with extensive heterochromatic regions (a 'megameric' chromosome) this element frequently lies alongside the X in the prophase of meiosis, or even appears to be stuck on to its side. In the bug *Edessa irrorata* (Schrader¹⁷⁹) the chromosomes contain extensive heterochromatic regions which tend to stick together during the prophase stages of meiosis in the male. Similar cases have been described in other groups of animals, and it would seem



TEXT-FIG. 1. Diagrams of a spermatogonial metaphase and the diakinesis stage of meiosis in a short-horned grasshopper such as *Mecostethus grossus*. Only two pairs of autosomes and the X-chromosome are shown (there would actually be eleven pairs of autosomes). *A* and *A* are a normal pair of autosomes, euchromatic except for a short region next to the centromere. *h* and *h* are a pair of autosomes with an extensive heterochromatic region and a shorter one at the other end of the chromosome. It will be seen that the heteropycnosis of the X is negative in the spermatogonial metaphase, positive at diakinesis. On the other hand, the heteropycnosis of the heterochromatic autosomal regions is only shown at diakinesis, when it is positive in character. (After White⁵⁴.)

that wherever extensive heterochromatic segments are present they will show a tendency to stick together. Whether this really demonstrates the existence of a force of attraction between them is much more doubtful, however. Some authors have regarded this fusion of heterochromatic regions as demonstrating some kind of genetic homology between the heterochromatic blocks, while others have spoken of it as a non-homologous association. In *Drosophila* species the main heterochromatic regions of all the chromosomes are joined together in the salivary gland nuclei to form the so-called chromocentre (see Section *g*).

The early geneticists soon discovered that (in *Drosophila* at any rate) heterochromatic regions possessed very little genetical activity. Thus the Y-chromosome, which is positively heteropycnotic at various stages of the nuclear cycle and which behaves as heterochromatin in the salivary gland nuclei (see Section *g*), is almost completely inert, genetically. In *Drosophila melanogaster* the proximal region of the X-chromosome is also heterochromatic and 'inert', and so are short regions in the middle of the 'second' and 'third' chromosomes, as well as certain portions of the tiny 'fourth' chromosome. Numerous much shorter

heterochromatic sections are scattered throughout the euchromatic regions, according to the evidence of the salivary gland chromosomes (see Section *g*).

It now seems probable that the association of heterochromatin and genetical inertness is a very general one which possibly occurs in all animals and plants—a fact which is of some use to the geneticist, since it means that he can predict the genetically active and inert regions of the chromosome set from a rapid cytological examination of his material instead of having to wait for the results of a complete genetical analysis. We may predict, for example, that the ‘megameric’ autosome found in many of the short-horned grasshoppers is mainly inert.

In the case of the Y-chromosome in *Drosophila melanogaster* we know that males lacking it (so-called X0 males) are quite viable, but they are sterile owing to their sperms being non-motile.* By studies on flies lacking parts of the Y, rather than the whole chromosome, it has been shown that there are several genes situated in the Y, all of which are necessary to ensure full motility of the sperm. The Y also seems to contain the wild-type allele of the *bobbed* gene. It has been shown by cytological studies that in the salivary gland nuclei (see Section *g*) the Y-chromosome shows about eight rather indistinct bands and it is probable that each of these corresponds to a gene, most of these genes being ones whose effects are only manifested in the sperm.

In addition to its effect on sperm motility, Mather¹²⁷ has claimed that the Y-chromosome of *D. melanogaster* carries genes which affect the number of sternopleural bristles of the fly. He believes that the heterochromatin carries large numbers of genes whose individual effects are slight but cumulative, which he calls *polygenes*, in contrast to the ‘major’ genes which produce large-scale effects. A somewhat similar standpoint is taken up by Pontecorvo¹⁶³ who, following Caspersson (see Caspersson and Santesson⁴²), regards each heterochromatic segment as built up of a large number of identical or at least very similar genes, thus leading to a condition in which its genetic effects are non-specific, or at any rate much less specific than those of euchromatic regions. This is in accordance with the well-known fact that deletions and duplications of heterochromatin are, in general, much less harmful than deletions or duplications of euchromatic segments of about the same size. It also agrees well with the observation that closely related species of *Drosophila* sometimes possess very different amounts of heterochromatin (Wharton²¹⁶; White²²⁰).

Can we regard the Y-chromosome of *Drosophila melanogaster* as completely heterochromatic? Possibly—but until the term can be given a more precise definition the question must remain open. It seems likely that there are no completely euchromatic chromosomes, i.e. that all

* Apparently the X-bearing sperms of X0 males are also non-motile.

chromosomes generally regarded as euchromatic and genetically 'active' contain some heterochromatic and 'inert' segments; but the converse proposition, that all heterochromatic chromosomes contain euchromatic regions, is not necessarily true. But the criteria for distinguishing heterochromatin are not precise enough, and if a chromosome is heterochromatic for nine-tenths of its length, the remaining one-tenth consisting of short euchromatic segments, it would probably be described as 'totally heterochromatic' except in an organism possessing salivary gland chromosomes, where a more detailed type of analysis is possible.

It is not easy to understand why genetically inert chromosomes should have persisted in the course of evolution. Most probably all heterochromatic segments and chromosomes possess 'residual' genetic functions such as have been shown to exist in the case of the Y-chromosome in *Drosophila*. Caspersson and Schultz⁴³ claimed that the oocytes of XXY females in *Drosophila* contained larger quantities of cytoplasmic nucleotides than those of normal XX females. Taken in conjunction with Bridges's³⁴ claim that the bands of the salivary gland chromosomes of XXY females stain more intensely than those of XX larvae, this would seem to suggest that the Y may elaborate 'extra' nucleic acids which are passed on to the other chromosomes and to the cytoplasm. But Callan³⁶ has recently disputed the claim of Caspersson and Schultz, having been unable to find any difference in the nucleotide content of the eggs of XXY and XX females, using an improved technique, and other workers on salivary gland chromosomes have failed to confirm Bridges's conclusion. It would thus appear that we should look to the specific properties of heterochromatin rather than to its general biochemical properties for an explanation of the survival of heterochromatic regions and chromosomes in evolution.

In certain species of both animals and plants so-called supernumerary chromosomes are present in some individuals but not in others, the number of supernumerary elements frequently varying very greatly from one individual to another. In some cases these supernumerary chromosomes seem to be derived from the sex-chromosomes. Thus in the bed bug the number of heterochromatic X-chromosomes varies from 3 to 15 (Slack¹⁹¹; Darlington⁵²). It is probable that only two or three of these really have a sex-determining function, the others being inert or nearly so. They may, however, possess slight genetic properties which have prevented them from being lost in the course of evolution. In the maize plant certain supernumerary chromosomes (known as B-chromosomes) may be present in some strains, where they vary in number from 0 to about 30; high numbers of B-chromosomes seem to diminish the viability and fertility of the plant, according to Randolph¹⁶⁵, so that they cannot be accumulated indefinitely.

A special type of heteropycnosis occurs in the males of certain scale-insects, where one entire haploid set of chromosomes is heteropycnotic (Schrader^{175,176}; Hughes Schrader⁸⁹; Thomsen²¹²). This heteropycnosis of half the somatic chromosome set apparently exists in all the tissues as well as during spermatogenesis. Some genetic evidence (Dickson⁶¹; see also White²²¹) indicates that it is probably the chromosomes of paternal origin which behave in this manner. They have presumably been 'preconditioned' by their passage through the sperm, so as to render them heteropycnotic in a male but not in a female scale-insect. Clearly, heteropycnosis cannot be an indication of genetical inertness in this case, since chromosomes which are non-heteropycnotic in one generation will give rise, in the course of successive divisions, to chromosomes which are heteropycnotic in the next generation. It is possible, however, that these scale-insect chromosomes contain extensive heterochromatic (= inert) segments and that these show such a degree of heteropycnosis in the case of paternal chromosomes in a male individual that the euchromatic regions are obscured. An analogous case, where chromosomes which are undoubtedly genetically 'active' show intense positive heteropycnosis, occurs in the spermatogonia and oogonia of certain gall-midges (White^{223,224}).

d. Spiral Structure of Chromosomes

Spiral structure was first observed in chromosomes of the plant *Tradescantia* by Baranetsky¹² in 1880, but it was not until much later that it was realized that this is the normal structure of all late prophase and metaphase chromosomes and presumably also for those chromosomes and chromosomal regions which remain 'condensed' during the resting stage. The cylindrical appearance of the chromosomes (or chromatids) at metaphase is thus a deceptive one, each apparently solid rod being really a tightly coiled spiral. Thus the thread along which the genes are spaced is in reality a good deal longer than the metaphase chromosome. The spiral is a compact one, in which there is virtually no space left between the gyres or in the centre of the coil, so that the usual methods of fixation and staining are inadequate for revealing the spiral structure except in particularly favourable cases, and special techniques have to be employed. These either lead to an artificial separation of the gyres or dissolve away a certain amount of 'matrix' material covering them.

In 'smears' of pollen mother-cells appropriately stained the spiral structure of the meiotic chromosomes can sometimes be seen without any special treatment, but some form of pre-treatment (i.e. exposure of the cells to a chemical agent before fixation) is usually necessary in order to reveal the details of spiral structure. Kuwada and Nakamura¹⁰⁹ used ammonia vapour, Oura¹⁵³ used solutions of potassium cyanide, while

other workers have used boiling water (Sakamura¹⁷²), desiccation (Huskins and Smith⁸⁶), or immersion in distilled water (Matsuura¹³¹). Most of the work has been carried out on plants with particularly large chromosomes, notably *Tradescantia*, *Trillium*, *Fritillaria*, *Rhoeo*, and the ferns *Osmunda* and *Todea*, but spiral structure can also be seen in plants with much smaller chromosomes, such as wheat (Huskins⁸²). In animal material it is much harder to demonstrate spiral structure in the chromosomes, but it has been seen in mitotic and meiotic chromosomes of many species of grasshoppers (Makino¹²²; White²¹⁹; Coleman⁴⁷; Geitler⁷³).

The whole cycle of mitosis can be regarded as a cycle of spiralization and unspiralization, a new coiling-system being formed in the protein framework at each division. This coiling develops during prophase. At first the gyres are very numerous and of small radius; as prophase develops they decrease in number as their radius increases, so that the condensed metaphase chromosomes have relatively few large coils; the number of coils will be $2l/d$ in a compact metaphase chromatid, where l is the length and d the diameter. Usually the coils of one mitosis persist to some extent through the resting stage, so that they can still be recognized as loose 'relic spirals' in the prophase of the next division, superimposed on the new coils which are forming within the chromatids. These relic spirals can be seen very clearly in the spermatogonia of some of the long-horned grasshoppers (Tettigoniidae); a good idea of their appearance can be gained from Plate 2, Figs. 1 and 2. They disappear before metaphase, the new coiling-system having fully developed by that time.

At meiosis each of the chromosomes of which the bivalents are composed is coiled in a wide-gyred spiral. The two chromatids are coiled together and may, in addition, be twisted around one another. It was long ago pointed out by Kuwada that if two threads are coiled in such a manner that they run strictly parallel throughout their length, they cannot be separated without uncoiling, since they will be interlocked at each turn of the spiral. On the other hand, if each thread is relationally coiled around the other at each turn of the spiral no interlocks will be produced and the threads can separate freely from one another. Kuwada¹⁰⁸ calls the former type of spiralization *orthospiral*, the latter *anorthospiral*; Matsuura¹³² uses the terms *relational* and *parallel* in the same sense, while Sparrow, Huskins, and Wilson¹⁹⁸ and Huskins⁸² speak of *plectonemic* and *paranemic* coils. Usually, sister-chromatid coils at the first meiotic division are paranemic, so that the chromatids can separate easily at the anaphases of the two meiotic divisions. At least, this is the case in *Trillium* (Huskins⁸²; Matsuura¹³²). In *Tradescantia*, however, plectonemic coiling of sister-chromatids seems to occur, according to the observations of Shinke (Kuwada¹⁰⁷). It may be

significant in this respect that in *Trillium* there is little terminalization of chiasmata between diplotene and first metaphase, and little or no uncoiling of the chromatids occurs between the two meiotic divisions; while in *Tradescantia* the chiasmata have moved to the ends of the bivalents before the coiling of the sister-chromatids is fully developed, and the coiling is at least partly unwound during the interkinesis stage between the two meiotic divisions. Thus we can understand why in *Trillium* paranemic coiling of sister-chromatids is necessary for them to be able to separate freely at the meiotic anaphases, while in *Tradescantia* plectonemic coiling is possible, since the coils are unwound before they separate and are replaced by a fresh set of gyres.

In mitotic divisions each chromosome or chromatid only possesses a single spiral, but in the chromosomes of the first meiotic division in some plants the thread which forms the 'major' spiral seems to be itself coiled in a 'minor' spiral, so that the whole structure resembles the 'coiled-coil' filaments of certain electric lamps. In most cases the major coil of the first meiotic division disappears during the interkinesis, the minor spiral becoming the main, and perhaps the only, system of coils present during the second meiotic division. It should be emphasized that the 'minor' spiral of the first meiotic division can only be seen under exceptionally favourable circumstances in plants with very large chromosomes (it has never been demonstrated in animal chromosomes), that the distance between its gyres is only just resolvable under the microscope, and that there is no reason to believe a minor spiral exists in all organisms. Some workers describe a 'waviness' or 'zig-zag' of the chromonema rather than a true minor spiral. Oura¹⁵³ has, however, published a remarkably convincing photograph of the minor spiral in *Tradescantia*, so that there is no reason to doubt its existence in some organisms, at any rate.

It was for some years a matter of controversy whether the direction of coiling (right- or left-handed) was constant or at random for any particular chromosome or chromosomal region. There is now overwhelming evidence, both for plant and animal chromosomes (Matsuura^{130,131}; Nebel¹⁵¹; White²¹⁹), that it is at random. Frequently the direction of coiling may be reversed at the centromere (Sparrow¹⁹⁷), and reversals have also been observed by some workers at other points along the length of the chromosome (Huskins and Wilson⁸⁸; Sparrow, Huskins, and Wilson¹⁹⁸; Matsuura¹³²; White²¹⁹). The fact that the direction of coiling in any particular chromosome is not constant is, incidentally, evidence that a new set of spirals is formed at each mitosis, since if the same spiral persisted from division to division we should expect that all the descendants of a single chromosome should be coiled in the same direction. It has been shown, however, that a cyst of 32 or 64 grasshopper spermatogonia, all derived from a single ancestral cell in the

course of five or six divisions, may contain an approximately equal number of right-handed and left-handed X-chromosomes (White²¹⁹).

Both euchromatic and heterochromatic regions of chromosomes are spirally coiled, but since the number of coils per unit of length is inversely proportional to the diameter of the chromosome the heterochromatic regions will possess more coils (of smaller amplitude) when negatively than when positively heteropycnotic.

e. Chromosome Reproduction

The ability of the chromosomes to reproduce themselves is one of their most remarkable characteristics, although it is a property which is also shown by virus particles and probably also by various types of self-duplicating particles in the cytoplasm, such as the kappa-substance of *Paramecium* (Sonneborn^{195,196}).

On physico-chemical grounds the duplication of a body composed of nucleo-protein molecules is believed to consist in the formation of 'copies' of the original molecules alongside the original or 'model' molecules. In general, the copies must be exact replicas of the models, but 'errors' in replication have been postulated as the physico-chemical basis of gene-mutation. Recently Prokofieva-Belgovskaya¹⁶⁴ has put forward a general theory of chromosomal reproduction, according to which 'daughter' chromosomes exhibit quite general differences (e.g. in staining capacity) from 'mother' chromosomes. Her evidence has, however, been criticized by Lortz¹¹⁶ and Muller¹⁴⁷, and her ideas seem unlikely to gain general acceptance (Kaufmann⁹⁸). It is, in fact, quite uncertain whether the two chromosomes which are derived from one at cell division are related as 'mother' and 'daughter'; possibly each contains both 'model' and 'copy' molecules.

At what stage in the nuclear cycle do the chromosomes reproduce? No cytological problem has been more hotly debated than this one. The earlier evidence was purely morphological. At metaphase, and even in prophase, chromosomes are visibly divided into two spirally coiled threads or *chromatids*, which lie roughly parallel to one another and are apparently joined together at the centromere. These spiral chromatids may be pressed into one another so that no space can be seen between them, or they may be separated so that there is a visible split down the length of the chromosome. But at what stage does this split occur? Darlington and his followers have claimed that it takes place during the resting stage, when the chromosomes are not in a suitable condition for accurate observation, being more or less 'unfixable'. Thus they believe that telophase chromosome threads are always single, prophase ones always double, whereas most other workers believe that telophase chromosomes are already split once and that prophase chromosomes are split twice, i.e. that each chromatid consists of two half-chromatids or

subchromatids, between which a secondary split can sometimes be observed. Among those cytologists who favour the latter view are Schrader¹⁸¹, Huskins⁸³, Manton¹²⁴, and Kaufmann⁹⁸. Nebel¹⁵² has even claimed that telophase chromosomes are quadruple, the split corresponding to each mitosis having taken place two divisions before. In the case of the meiotic divisions, Darlington believes that the split is delayed, or, as he puts it, that the onset of prophase is precocious, i.e. it begins before the chromosomes have split. He thus claims that in the earliest prophase stages of the first meiotic division (leptotene and zygotene) the chromosome thread is single and that the split into chromatids does not take place until the pachytene stage. Other authors (e.g. Huskins and Smith⁸⁶) have published convincing figures of already-divided leptotene chromosomes in various species of plants, and Hughes Schrader⁹⁰ has shown that in the scale-insect *Llaveiella* the meiotic bivalents are visibly octopartite, i.e. they possess three 'splits'—a primary one corresponding to the plane between the maternal and paternal chromosomes, a secondary one between the chromatids, and a tertiary one between the half-chromatids. In this case the half-chromatids even possess separate spindles.

Attempts have been made to resolve this controversy by experimental means (Mather and Stone¹²⁸; Mather¹²⁶). It has been argued that X-rays will only break single strands and that if an anaphase or telophase nucleus is irradiated one will observe 'chromosome-breaks' in the following division if the chromosomes were unsplit at the time of irradiation, 'chromatid-breaks' if they were split. Mather¹²⁶ applied this technique to pollen-grain mitoses in various species of plants, and obtained results in apparent disagreement with those reported earlier by Huskins and Hunter⁸⁵, using the same type of material. It is somewhat doubtful, however, whether one is really justified in assuming that irradiation will never break two strands of a chromosome at the same level. Thus Lea¹¹¹ concludes that: 'If the X-ray experiments indicate that the chromosomes are split, this can be relied upon, but X-ray evidence for a chromosome being unsplit is not final.'

A somewhat different type of experimental evidence bearing on the question of the time of division of chromosomes has been brought forward by McClintock¹¹⁹ who observed the behaviour of maize chromatids which were broken at meiotic anaphases by stretching on the spindle, not by irradiation. She found by observations on the chromosomes at the next division that 'each broken chromatid behaves as if it were split, with fusions occurring between its two longitudinal halves at the position of breakage'. This would agree with her observation that at the diakinesis stage in maize the chromatids can sometimes be seen to be divided into half-chromatids by a 'tertiary split'.

Most of the cytologists who have debated this question of the time

of splitting of chromosomes seem to have tacitly assumed that this time must be the same in all types of cells. This seems rather unlikely; at any rate, we have no right to assume that the split always occurs at the same stage of the nuclear cycle, when it is known that in some cells the resting stage is very prolonged, while in others there is a much more direct transition from the telophase of one mitosis to the prophase of the next. It is clear, however, that the overwhelming majority of cytologists are now opposed to Darlington's view as to the time of splitting of chromosomes (Schrader¹⁸¹; Huskins⁸³).

When we consider the problem of chromosomal reproduction from the chemical standpoint, it is possible that the exact time when the split becomes visible may not be of any great importance, since it is highly probable that the essential process of genic duplication may occur long before any split is visible under the microscope; in fact, it is even possible that all chromosomes are in reality bundles of submicroscopic gene-strings. Very recently Huskins⁸³ has claimed that under abnormal circumstances a chromosome may split into three instead of two chromatids, but the evidence on which this conclusion is based has not yet been published.

That chromosomes may be caused by experimental agencies, such as X-rays and abnormal temperatures, to undergo an extra split, so that they are quadruple at stages of mitosis when they would normally be double, has been shown by various workers (White²¹⁷; Barber¹³). It is possible that this condition sometimes results from the suppression of a mitosis which would otherwise have occurred, the split which would have corresponded to that division not being inhibited. The condition is probably akin to endopolyploidy (see Section *f*) and may in some cases be regarded as an experimentally produced endomitosis, perhaps of a special type. Sometimes diplo-chromosomes (i.e. two chromosomes united by an undivided or imperfectly divided centromere) persist for some time, but they eventually separate into two independent chromosomes. In 'sex-ratio' males of *Drosophila pseudoobscura* and some related species the X-chromosome splits twice instead of once, as it does in the meiosis of a normal male (Sturtevant and Dobzhansky²⁰⁵).

If the chromosome thread is spirally coiled at the time when it splits, the split must, of course, follow a spiral course, parallel to the axis of the thread. There is some evidence, however, that this parallelism may sometimes be imperfect. We mentioned earlier that endless 'ring-chromosomes' suffered from various types of accidents at mitosis. In maize it has been shown by McClintock^{118,120} that ring-chromosomes in the somatic tissues of the sporophyte frequently give rise to double-sized rings, containing two centromeres, instead of to two rings, each with one centromere. When the two centromeres of a double-sized ring pass to opposite poles at anaphase the portions of the chromosome

between them become stretched on the spindle and eventually broken. Thus each daughter-cell receives a V-shaped chromosome with one centromere and two broken ends. Since the positions of breakage are not constant, the sizes of the Vs and the relative lengths of their arms vary greatly. The two freshly broken ends of each V soon fuse together, so that rings are produced in the daughter-nuclei, but these rings are not necessarily the same size as the parent ring and ordinarily contain deficiencies or duplications of genetic material; in many such rings both duplications and deficiencies will be simultaneously present. The presence of a ring-chromosome in a maize plant thus leads to much mosaicism, patches of somatic tissue lacking some genes and possessing others in duplicate. The ring-chromosomes known in *Drosophila melanogaster* seem to be somewhat more stable, but they probably also produce double-sized rings in some cells.

It is easy to understand that the formation of a double-sized ring might result from the plane of the split making one complete turn around the plane of the chromosome, and this is the most probable explanation; but it is, alternatively, possible that some kind of somatic sister-strand crossing-over occurs. The phenomenon depends on the size of the ring, the larger rings being much more unstable than the smaller ones.

In some special cases a failure of certain of the chromosomes to split normally at mitosis may lead to their being left 'helpless' in the middle of the spindle at anaphase, while the remaining chromosomes pass to the poles. In this way, in the embryos of certain insects, some of the chromosomes are eliminated from those nuclei which are destined to pass into the somatic cells, so that the somatic nuclei come to contain fewer chromosomes than those of the 'germ-line'. This is the case in some species of midges of the genus *Sciara*, where the spermatogonia and oogonia each contain several large 'limited' chromosomes which are entirely absent from the somatic nuclei (Du Bois⁶²; Metz¹³³). The centromeres of these chromosomes seem to function normally up to and during the metaphases of the elimination divisions, but the chromosomes do not seem to have split except in the neighbourhood of the centromeres, or else their chromatids are unable to separate from one another, so that when the daughter-centromeres begin to separate on the elongating anaphase-spindle they are held together by the arms of the chromosomes which cannot separate from one another. The 'limited' chromosomes thus remain stretched in the middle part of the spindle and do not get included in either of the daughter-nuclei: they remain in the yolky egg cytoplasm for some time, but eventually degenerate, being presumably broken down by cytoplasmic enzymes. In the cleavage divisions of the primordial germ-cell nuclei, which lie in the polar-plasm at the posterior pole of the egg, no elimination of limited chromosomes normally occurs

—presumably the cytoplasmic environment in which they lie permits them to split normally and thus preserves them from the fate which overcomes their 'sisters' and 'cousins' in the somatic nuclei.

A similar elimination of some of the chromosomes takes place in the gall-midges (Cecidomyiidae) where it seems to be characteristic of the whole family (Kahle⁹⁵; Kraczkiewicz^{105,106}; Reitberger¹⁶⁶; White^{222,223,224,225}). Thus in *Miastor* the male somatic nuclei contain 6 chromosomes and are haploid (except in those tissues where endopolyploidy occurs), the female somatic nuclei show 12 chromosomes and are diploid (again, with the exception of the endopolyploid tissues), while the germ-line nuclei have 48 chromosomes (White²²³). Here the male embryos eliminate 42 chromosomes from the somatic nuclei during certain of the early cleavage divisions, while the female nuclei only eliminate 36. In another Cecidomyid, *Taxomyia taxi*, there are 6 chromosomes in male somatic cells, 8 in those of females, and 40 in germ-line nuclei of either sex; male embryos eliminate 34 chromosomes from the somatic nuclei, female ones 32. The mechanism of elimination seems to be exactly as in *Sciara*, although the number of chromosomes eliminated is much greater.

The role of these 'limited' chromosomes, which are confined to the germ-line, in the genetic system of the species is far from clear. Certain species of *Sciara* lack limited chromosomes altogether, while in others a pair are regularly present. Throughout the whole family Cecidomyiidae, which includes many thousands of species, limited chromosomes seem invariably to be present, and are always far more numerous than the chromosomes in the somatic nuclei. Genetic evidence suggests that the limited chromosomes of *Sciara* are more or less inert, but it is probable that they possess some 'residual' genetic properties. In the Cecidomyids the fact that they occur in all species indicates that they have some function, although in the absence of genetic evidence one cannot be certain of this; it has recently been suggested that when they are broken down and their substance is dispersed through the yolky egg cytoplasm, they do not cease to function but continue to influence embryonic, and perhaps even larval, development for some time (White²²⁵).

The reason why the chromatids of the limited chromosomes are unable to separate properly at the time of elimination is not clear. It is, indeed, uncertain if this is the proper interpretation—possibly the chromosome thread has never split into chromatids along the whole of its length. Alternatively, it is possible that the two chromatids are coiled together, plectonemically, so that they are interlocked at each gyre of the spiral. A failure of the chromonema to divide has apparently been induced in certain plant chromosomes by the application of unusually low temperatures (Darlington and La Cour⁵⁶). In this instance the failure to divide occurs only in certain chromosomal regions which are

deficient in nucleic acids (or at any rate in stainable material) in the chilled plants. The appearance of such regions of 'differential reactivity' (Wilson and Boothroyd²²⁸) or 'olistherozones' (Resende *et al.*¹⁶⁷) may be caused by various other factors besides low temperatures. Darlington and La Cour have regarded it as simply due to 'nucleic-acid starvation', but the phenomenon is probably more complex than this, although such regions probably are deficient in nucleic acid as compared with other chromosomal regions.

If it is true (1) that regions of 'differential reactivity' have little nucleic acid and (2) that splitting into chromatids is incomplete in such regions, then it is highly probable that a causal connexion exists, namely, that the normal process of splitting depends on a certain minimum amount of nucleic acid, and that when a subminimal amount is present the split will not occur. But it must be remembered that the negatively heteropycnotic X-chromosomes of grasshoppers (see p. 196) and of certain mammals (Koller^{101,102}) split normally, and at the same time as the other euchromatic chromosomal regions.

f. Endopolyploidy

The statement made in most elementary accounts that all the nuclei of an organism contain the same number of chromosomes is only a first approximation to the truth. Its frequent reiteration has, however, blinded many biologists to the fact, which has long been known (although its significance has only recently become apparent), that many of the differentiated cells of an adult organism are polyploid, that is to say, they contain 2, 4, 8, 16... times the diploid ($2N$) number of chromosomes. This phenomenon is, in general, only exhibited by the nuclei of cells which have differentiated so far that they have lost the power to divide by mitosis, unless reactivated to do so by some special treatment (e.g. by growth-hormones in the case of plants). The phenomenon thus naturally passed unnoticed so long as cytologists only counted the chromosomes of cells in mitosis. Recognition of the very general occurrence of 'endopolyploidy' in both the plant and animal kingdoms has resulted from the development of techniques whereby the chromosomes can be counted in resting nuclei (Geitler^{69,71,72}) or whereby nuclei which would not otherwise divide again are caused to do so.

In certain cases, however, these polyploid somatic cells may continue to divide by mitosis. Thus, as early as 1910 Stomps²⁰⁴ found diploid, tetraploid, octoploid, and occasionally 16-ploid metaphases in root tips of *Spinacia oleracea*, a phenomenon to which Langlet¹¹⁰ applied the term 'polysomaty'. These have been reinvestigated by Gentcheff and Gustafsson⁷⁴, who found that the doubling of the chromosome number resulted from a double reproduction of the chromosomes during the resting stage.

In the ileum of mosquito larvae the epithelial cells do not multiply

during larval life but they increase in volume unequally, so that when the larva has finished growing they are of several different sizes. It has been shown by Berger^{28,29} that the nuclei of these cells remain in a resting-stage condition during the whole of larval life, but that during the pupal period they begin to divide again. During the prophases of these divisions each nucleus contains six large bundles of chromonemata (the haploid number is three in mosquitoes). At the end of prophase these six bundles fall apart into their constituent threads, which we may now consider as independent chromosomes. There are usually 48 or 96 of these chromosomes, so that the nuclei have become 16- or 32-ploid during the long larval resting stage. These chromosomes become aligned in pairs just before metaphase, and at anaphase a whole chromosome of each pair goes to either pole. Thus, a 32-ploid cell gives rise to two 16-ploid ones. This process of somatic reduction is repeated several times during the pupal metamorphosis, so that by the time the metamorphosis is completed the cells are diploid or tetraploid (Berger^{28,29}; Grell⁷⁵).

These cells of the mosquito ileum thus show (1) chromosomal multiplication without mitosis (during larval life) and (2) somatic reduction divisions (during pupal life). The latter process is clearly made possible by the somatic pairing phenomenon so characteristic of the Diptera, and the 'bivalents' which are formed never show any chiasmata: they are hence comparable to the meiotic bivalents of the male *Drosophila*, in which no crossing-over occurs.

Another instance of a tissue in which chromosomal reproduction without mitosis leads to the development of polysomaty is in the testicular envelope of the short-horned grasshoppers, where flat, pancake-shaped nuclei containing 23, 46, and 92 chromosomes occur (Plate I, Fig. 3). Here the chromosomes never become as fully condensed as in a true metaphase but they do go through cycles of condensation and decondensation which seem to correspond to the division cycles. Geitler⁷¹ has also described what he calls 'endomitotic' cycles of condensation and decondensation during the formation of endopolyploid nuclei in the Heteroptera, and Painter and Reindorp¹⁵⁸ have described similar stages in the development of the nurse-cells of *Drosophila melanogaster*, which eventually become 512-ploid. The use of the terms 'endoprophase', 'endometaphase', &c., to describe the various stages of the endomitotic cycle is perhaps unfortunate, since there is never any strong metaphase condensation, the chromosomes remaining 'fuzzy' throughout the whole process. No spindle is formed, the nuclear membrane never breaks down, and the split halves of the chromosomes simply drift apart, there being no active anaphase movement. The fact that in endomitosis (which we may probably regard as a highly modified mitotic process) cycles of condensation and decondensation still occur, suggests

that condensation has something to do with chromosomal reproduction, but the exact relationship is not clear.

That endomitosis may give rise to very high degrees of endopolyploidy has been shown by Geitler^{69,71,72} in the case of the pond-skaters of the genus *Gerris*. Here the diploid set of the male consists of twenty autosomes and an X which is heterochromatic. The individual chromosomes can be counted in many of the somatic nuclei, and in some species the X's can be made out very clearly on account of their heteropycnosis. Geitler found that particular tissues showed characteristic degrees of polyploidy. Thus, the nuclei of the tracheal epithelium were diploid or tetraploid, those of the fat body tetraploid, octoploid, and 16-ploid; those of the oenocytes up to 128-ploid, while in the salivary glands Geitler found giant branching nuclei which were estimated, from their volume and from the number of X-chromosomes, to be 512-ploid, 1,024-ploid, and even 2,048-ploid. It was naturally impossible to count the chromosomes in these nuclei (a 2,048-ploid nucleus should have contained 21,504 chromosomes in a male, 22,528 in a female!). It seems likely that the great branching nuclei in the spinning glands of caterpillars and caddis-fly larvae (Vorhies²¹⁵) are of the same general type as those of *Gerris*, the nucleus increasing in size and branching out into the cytoplasm as the number of chromosomes within it is repeatedly doubled.

In those insects where most of the larval tissues undergo histolysis during the pupal stage, the adult organs arising from 'imaginal buds', it is clear that the imaginal bud cells do not become endopolyploid and that they retain the ability to divide by true mitosis. The neurocytes also seem to remain diploid, since whenever they divide by mitosis in nymphal or late larval instars they always show the diploid number of chromosomes.

Most studies of endopolyploidy have been carried out on plant or insect material, but it seems clear that the phenomenon also occurs in other groups of organisms. In the Protozoa the macronuclei of the Ciliata are almost certainly endopolyploid, although chromosomes usually cannot be seen in them and although they divide by true amitosis. In the Vertebrates it was shown by Jacobi⁹⁴ that certain somatic tissues show several different size classes of nuclei, whose volumes bear a fairly definite relationship to one another. It is tempting to conclude that these represent an endopolyploid series (e.g. diploid, tetraploid, and octoploid nuclei in mammalian liver). But the cytological conditions are much less favourable for a detailed analysis than they are in insects and plants. In Invertebrates other than insects few studies of endopolyploidy have been carried out.

In insects it is clear that endopolyploidy and polysomaty play an important part in histological differentiation. But it is not clear whether

a cell in *Gerris*, for example, becomes an oenocyte because it is 128-ploid, or whether it becomes 128-ploid because it is an oenocyte. The latter seems much more probable, but experimental studies on histological differentiation in relation to endopolyploidy are badly needed. The highest degrees of endopolyploidy always seem to be found in gland-cells, or at any rate in cells which are producing large quantities of protein materials, and Painter¹⁵⁷, as a result of his studies on salivary gland chromosomes and on the lateral pharyngeal gland of worker bees, has concluded that there is a causal relation between endopolyploidy and protein formation, and he is disposed to attach particular importance to the multiplication of heterochromatic regions and nucleolar organizers which occurs in endopolyploid nuclei.

The observations of Huskins and his collaborators^{83,84} on polysomaty in plant material (roots of *Rhoeo* and barley) differ in some respects from those of previous workers. These authors used strong doses of indole-acetic acid to cause cells which would not normally divide again to enter upon mitosis. In addition to diploid, tetraploid, and octoploid cells they report the finding of 2 haploid cells, 2 (?) pentaploid ones, and 2 hexaploid ones, out of a total of 2,866 nuclei analysed in *Rhoeo* and 1,496 in barley. They also claim to have observed somatic reduction divisions, i.e. cells in which the chromosomes were paired to form true bivalents. Since the full account of this work has not yet appeared a critique of it would be premature, but it may be pointed out that diplochromosome formations in somatic cells could easily be mistaken for bivalents if extreme care were not exercised. The finding of occasional pentaploid and hexaploid nuclei in somatic cells (which could not, of course, have arisen by endomitosis or any similar phenomenon) would be explained if, occasionally, distinct cells underwent fusion, as sometimes happens to insect spermatocytes, possibly under the influence of abnormal local conditions.

g. Salivary Gland Chromosomes

A very special kind of endopolyploidy seems to be responsible for the production of the 'salivary gland' chromosomes in the larvae of the dipterous flies. These are giant chromosomes whose volume is many thousands of times that of ordinary mitotic chromosomes, and which appear somewhat like earthworms under the microscope, with numerous transverse bands of dark-staining material alternating with non-staining regions. Typically, each individual chromosome in the salivary gland nuclei is closely fused with its homologous partner, so that the elements appear to be present in the haploid rather than the diploid number. This is probably due to the somatic-pairing tendency which, as we have already seen, is characteristic of most dipterous chromosomes. As a matter of fact the closeness of fusion in the salivary gland

nuclei varies somewhat throughout the Diptera; in some forms it is complete, except for the X-chromosome, which is necessarily unpaired in the male, while in others the homologous chromosomes are only loosely paired, actual fusion being limited to a few short regions.

No spiral structure can be observed in salivary gland chromosomes, except that frequently the homologues are loosely wound round one another. It would seem that the absence of spiralization is one of the reasons for the great length of the salivary gland chromosomes, as compared with ordinary mitotic chromosomes. It can hardly be the only reason, however; the disparity in length is too great (salivary gland chromosomes may be 50–100 times the length of metaphase chromosomes). Owing to their great length the salivary gland chromosomes can only find room, even in the very large nuclei in which they occur, by being rolled up into a fairly tight tangle, which fills practically the whole of the nuclear cavity.

The term 'salivary gland chromosome' is rather unfortunate, since chromosomes of this type are found in many other somatic tissues of the Diptera. Thus Makino¹²³ found that in *Drosophila virilis* the nuclei of the oesophageal epithelium, the glandular cells of the proventriculus, of the intestinal epithelium, of the Malpighian tubules, and of the fat body all show chromosomes which approximate to the salivary gland type. There seems, in fact, to be a complete series of gradations in these and other tissues between normal somatic chromosomes and salivary gland ones, the degree of development of this type of chromosome being, in general, greater in the larger nuclei than in the smaller ones.

In making preparations of these chromosomes it is usual to crush the glands in a solution of 'aceto-carmine' or 'aceto-orcein' under a cover-glass, so that the nuclear membranes are ruptured and the chromosomes are stretched and spread out flat.

Originally discovered by Balbiani¹¹ and studied about the same time by Carnoy³⁷, who published some remarkably accurate figures of their structure, the salivary gland chromosomes aroused little interest until 1933, when Heitz and Bauer, in Germany, described their appearance in the fly *Bibio*, and Painter, in the U.S.A., having studied them in *Drosophila*, pointed out that they could be used for cytogenetical analyses of a type not previously employed. This possibility depends on the fact that the dark-staining bands are individually recognizable, the thickest ones being many times broader than the finest ones, which can only be seen in stained preparations of the highest quality under the most favourable optical conditions (Bridges³³), or using ultraviolet microscopy (Ellenhorn, Prokofieva, and Muller⁶⁴). Apart from the presence of inversions or other structural rearrangements, the bands are constant in position in any one species, so that it is possible to construct cytological 'maps', in which each band is referred to by number. These

maps constitute the basis of most modern cytogenetical work on *Drosophila*.

It has been proved by staining methods, ultraviolet spectroscopy (Caspersson³⁸), and micro-incineration (Barigozzi^{14,15}) that the bands are rich in nucleic acids, the internodes between them being practically free of this substance and hence presumably made of protein materials.

The work of Serra^{184,185,186} and Serra and Queiroz Lopes^{188,189}, using the α -naphthol-hypobromite test for arginine, confirmed the findings of Caspersson³⁹ based on ultraviolet absorption spectroscopy, that the proteins of the bands are, in the main, of the histone type (i.e. strongly basic and containing much arginine), while the proteins of the interband regions are of a higher type, presumably the tryptophane-containing protein of Mirsky and Pollister.

The bands of the salivary gland chromosomes rather evidently correspond to the chromomeres of the meiotic chromosomes. In both cases there is a tendency for neighbouring bands or chromomeres to be fused together, so that the number of 'ultimate' chromomeres or bands is only revealed when the chromosome is artificially stretched so as to separate them.

The bands of the salivary gland chromosomes are, of course, really 'disks', which extend right through the thickness of the chromosome. Many of them are obviously built up of a number of chromomeres or granules, all lying in the same plane (transverse to the long axis of the chromosome). It is probable that all the bands are in reality of this nature, but in many of them the chromomeres have fused into a continuous disk. In many cases fine 'fibres' can be seen extending from the chromomeres of one band to those of the next, although it has been claimed by some workers that these are artefacts.

In a number of instances a careful study of *Drosophila* stocks with minute deletions or other structural rearrangements has enabled cytogeneticists to localize the position of particular genes within single bands. Thus, in *Drosophila melanogaster* the Notch gene lies in the 3C7 band (Slizynska¹⁹²; Demerec⁵⁸), the Bar gene in the double band 16A 1·2 (Sutton²⁰⁶), and the star-asteroid gene (or genes?) in the double band 21E 1·2 (Lewis¹¹⁴).

Micro-dissection studies which have recently been carried out on the salivary gland chromosomes of *Chironomus* (d'Angelo⁴⁹) have shown that when the chromosome is stretched by means of needles it is the interband regions rather than the bands which undergo elongation: even after a tenfold elongation the chromosomes return to their normal length when the tension is released.

The total number of bands cannot be stated with certainty, since many of them are so fine ($0\cdot2\mu$ wide) as to be almost below the limit of resolution of the microscope, while some of the thicker ones may consist

of several bands situated so close together (i.e. with such short internodes between them) that they appear as a single one. The total number of bands which have been detected in the complete chromosome set of *Drosophila melanogaster* is 5,149 (P. N. Bridges³⁴; Slizynski¹⁹³), but it is probable that a reinvestigation would establish the existence of a good many more. If each band represents the physical basis of a single gene, it is obvious that a count of the total number of bands would indicate the total number of genes, which has been estimated at under 10,000 in *Drosophila* by various workers.

The exact relationship of the salivary gland elements to ordinary mitotic chromosomes has been very much debated. In development they arise from chromosomes which are clearly split into two chromatids and show a chromomeric structure. The homologous elements then undergo pairing, so as to produce a 4-stranded structure (Painter¹⁵⁵). This then undergoes a progressive increase both in length and in diameter. The chromomeres become the bands of the fully formed salivary gland chromosome, but it is evident that most of the chromomeres which are seen in the early stages are compound and that they separate into their constituent chromomeres as the chromosomes elongate.

As the chromosomes become broader one can see more and more chromomeres in each transverse band and more 'interband fibres' (Painter¹⁵⁵). Thus the development of the salivary gland chromosomes is consistent with the view first put forward by Koltzoff¹⁰³ and Bridges³² that they are really bundles of threads, the chromonema having divided repeatedly, so that we eventually have a structure something like a multi-stranded wire cable, each wire being enlarged periodically into a 'chromomere', all the chromomeres at any one level showing a tendency to fuse into transverse bands or disks. This is the so-called *polytene* (or many-stranded) theory of the essential nature of salivary gland chromosomes which is generally accepted to-day, although not by Metz¹³⁴, who has put forward a different interpretation, while willing to admit that the salivary chromosomes may be bundles of very many submicroscopic, and therefore invisible, chromonemata.

A new interpretation of the structure of the salivary gland chromosomes which was put forward by Ris and Crouse¹⁷¹ has not met with general acceptance. These authors have claimed that the chromonemata in the salivary gland chromosomes *are* spirally coiled and that the bands and chromomeres are optical illusions based on misinterpretation of coiled structures. Their views have been criticized by Hinton⁸¹ and by Kaufmann⁹⁸.

It is unfortunate that the salivary gland technique will never be applicable to organisms other than the dipterous flies, since chromosomes of this kind are unknown outside the order Diptera. It is clear, however, that the choice of *Drosophila* as material for genetical work

was a far more fortunate one than was realized before 1934, when salivary gland chromosomes first came into general use in genetical laboratories.

Even within the Diptera, however, the salivary gland nuclei vary greatly from one species to another. A variety of conditions have recently been described in the gall-midges (White^{222,226}). Thus in *Miastor* the nuclei of the larval salivary gland are endopolyploid rather than polytene. A particularly interesting case is that of *Dasyneura affinis*, where the salivary gland contains a proximal region (the 'basal reservoir') in which the nuclei are polytene (i.e. their chromosomes have the typical 'salivary gland' structure) and a distal region in which the nuclei are endopolyploid. At the junction of these regions a few cells are found which exhibit an intermediate condition, the bundles of chromonemata which represent the 'polytene' chromosomes being frayed out to give a condition which approaches the endopolyploid state of the more distal nuclei. This may be regarded as a striking proof of the reality of 'polyteny'.

Another particularly interesting case among the gall-midges is that of the genus *Lestodiplosis* (Plate 4). Here all the nuclei of the salivary gland are polytene, but in one particularly large cell situated in the proximal part of each gland they are present in a polyploid condition—that is to say, we have a nucleus which is both polytene and polyploid. Such a condition could arise if the chromosomes in the embryo and young larva first of all went through an endomitotic phase in which their constituent strands or chromonemata separated from one another and later continued to multiply chromonemata which failed to separate, so giving rise to the polytene condition (White²²²).

Although it has been known for a long time that the diameter of the salivary gland chromosomes varies along their length, there being characteristic enlargements alternating with thinner regions and occasional narrow constrictions (see Plate I, Fig. 4), few authors seem to have considered the interpretation of these differences in terms of the polytene hypothesis. Suppose that one could see the individual chromonemata in a transverse section of a salivary gland chromosome (which is not possible with existing techniques), would there be more of them in a region of the chromosome that has a larger cross-sectional area (a 'puff' or 'bulb') than in a region where the cross-sectional area is smaller (a 'waist')? If so, the chromonemata must branch and rejoin in the course of their length. Alternatively, it is possible that the chromonemata themselves vary in thickness along their length in the same way as the chromosome as a whole does, or that in the 'puffs' there is a greater amount of 'matrix' substance between the chromonemata than exists in the thinner regions. In the gall-midge *Camptomya* the salivary gland nuclei contain three chromosomal elements of normal dimensions and

one extraordinarily thick element (Plate 3), but there seems no reason to regard this as anything but an exaggeration of the differences in diameter which exist along the length of all polytene chromosomes.

We have not hitherto said anything about the condition of the heterochromatic regions in the salivary gland nuclei. In some species of *Drosophila* and in many of the gall-midges (White²²⁶) more than one type of heterochromatin can be seen in the salivary gland nuclei. In some cases we have heterochromatic bands which are intensely charged with stainable material, thus resembling the condition we have referred to as positive heteropycnosis in mitotic chromosomes, while in other types of heterochromatin the bands may be weak-staining, with their chromomeres only taking up the dye peripherally, so that they look 'hollow' under the microscope. The latter condition is possibly analogous to the negative heteropycnosis of some mitotic chromosomes.

In *Drosophila* the heterochromatic regions round the centromeres, together with the whole of the Y-chromosome, are all fused together to form a body known as the chromocentre, which consequently consists entirely of heterochromatin. All the heterochromatic regions are relatively much shorter in the salivary gland chromosomes than they are in ordinary mitotic ones. Thus the heterochromatic part of the X-chromosome of *D. melanogaster*, which forms about two-fifths of the total length of the metaphase chromosome, is represented by one-twentieth or less of the salivary gland chromosome.

The heterochromatic regions in the chromocentre contain a few bands, but these stain rather faintly, and the pairing of the bands is not very regular.

In most genera of Diptera other than *Drosophila* there is no chromocentre, all the chromosomes in the salivary gland nuclei lying free or only loosely attached by their ends, so that they are readily separated during the making of the preparation. The presence or absence of a chromocentre does not seem to be related to the presence or absence of heterochromatic regions, since many of the species without a chromocentre have extensive heterochromatic regions, either intercalary or terminal.

Not only are the proximal heterochromatic regions of the *Drosophila* salivary gland chromosomes fused together to form a chromocentre, the distal ends are also normally attached together more loosely, so that in making squashed preparations they are usually separated (Hinton⁸⁰), and Slizynski¹⁹⁴ has found that many of the short intercalary heterochromatic segments exhibit occasional pairing with one another.

The appearance of the salivary gland chromosomes is an extremely sensitive index of the nutritional condition of the larva. It has long been known that in order to obtain really good preparations of *Drosophila* salivary gland chromosomes the larvae need to be abundantly

supplied with yeast. The polytene chromosomes of mosquito larvae were long regarded as too thin and faint-staining to be of any use for a detailed analysis, but Frizzi⁶⁸ has shown that by supplying the larvae with a particularly rich diet of yeast and *Vorticella* the salivary gland chromosomes can be induced to grow to a much larger size, so that they are almost as suitable as those of *Drosophila* for the detection of inversions and other rearrangements.

It should be emphasized that nuclei of the salivary gland type only occur in cells which will never divide again by mitosis, nor has any experimental technique been discovered which will induce such cells to divide. The three forms of chromonematal reproduction—mitosis, endomitosis, and polyteny—represent alternative conditions. In mitotic reproduction the chromonemata that are formed as a result of nucleoprotein synthesis are segregated by a precise mechanism (the spindle) to different daughter-cells. In endomitosis the chromonemata separate, but in the absence of any mitotic mechanism they remain within the same nucleus, thus leading to the condition known as endopolyploidy. In polyteny the multiple chromonemata do not separate but remain permanently in bundles.

Why are chromosomes of the salivary gland type unknown outside the order Diptera? We believe that this question has not received a satisfactory answer. It has been suggested that the existence of the salivary gland type of chromosome is rendered possible by the 'somatic pairing' which is so characteristic of dipterous chromosomes. While it is undoubtedly true that it is the somatic pairing attraction which brings together the homologous chromosomes in the salivary gland nuclei, there is no reason to believe that it plays a part in preventing the hundreds or thousands of chromonemata in each polytene chromosome from separating, since these seem to be contained in a single matrix. And in the fully developed salivary gland nuclei the homologous chromosomes seem to be actually fused together, rather than merely held together by some force of attraction.

The largest chromosomes of the salivary gland type always seem to occur in the nuclei of cells which are active in manufacturing considerable quantities of protein, which is undoubtedly a clue to their physiological significance. But in the Cecidomyids there is a definite tendency for the very largest nuclei of all to be endopolyploid rather than polytene, the chromonemata falling apart as the nucleus develops to its maximum size.

h. Structural Rearrangements of Chromosome Parts

Chromosomes may undergo breakage (1) spontaneously, i.e. due to unknown causes, (2) as a result of stretching on the spindle at anaphase, (3) as a consequence of irradiation, (4) after treatment of the organism

with various chemical agents. The breaks produced in these different ways all seem to be of the same type, since they all behave similarly.

Spontaneous breakage only occurs very rarely, but Morgan¹⁴⁴ has described a case in which a structural rearrangement was present in two (possibly three) cells of a *Drosophila* salivary gland, but not in the remaining cells. Here the rearrangement had obviously occurred in one cell during the embryology of the gland. In certain special cases a chromosome may be liable to break spontaneously at a particular locus with a high frequency (McClintock¹²¹).

Breakage as a result of stretching on the spindle is liable to occur whenever two centromeres in the same chromatid pass to opposite poles at anaphase, the region between them being simply pulled out until it breaks. Ordinarily chromosomes with more than one centromere do not occur naturally, but they may arise as a result of spontaneous or induced rearrangements or in ring-chromosomes which have 'mis-divided' so as to produce double-sized rings with two centromeres.

Freshly broken chromosome ends which have arisen in any of the various ways enumerated above tend to join up with other freshly broken ends: they will not, in general, fuse with 'natural' chromosome ends (telomeres). Any freshly broken end, whether heterochromatic or euchromatic, will fuse with any other, so that all possible types of structural rearrangement can occur, although only chromosomes containing one centromere and two telomeres (or no telomeres, in the case of a ring-chromosome) are able to persist through a number of mitoses.* Muller¹⁴⁶ has pointed out that since freshly broken ends all seem to be of the same kind, it is unlikely that breakage occurs within a polypeptide chain, since this would be expected to give rise to 'positive' and 'negative' ends.

It is easy to understand why chromosomes lacking centromeres are inviable. Not being attached to the spindle at mitosis, they simply float about in the cytoplasm and usually fail to become included in either of the two daughter-nuclei at telophase. A chromosome which remains for any length of time in the cytoplasm between mitoses always seems to disappear eventually, being probably dissolved by enzymatic action.

As a consequence of the principles enumerated above it is evident that before a structural rearrangement can occur in a nucleus two or more breaks must occur, since a single break only produces two 'sticky' ends, and these can only rejoin in such a way as to restore the original sequence. On the other hand, if the two chromatids of a chromosome are both broken at the same level (a 'chromosome-break' as opposed to a 'chromatid-break') a process known as *sister-strand reunion* may occur, so as to produce a dicentric chromatid and an acentric one, each consisting of two homologous portions joined together.

* Catcheside and Lea⁴⁶ do not accept the 'telomere hypothesis', and believe that both terminal inversions and terminal deletions can occur and be viable.

We have no idea how often single breaks occur spontaneously, but the frequency of spontaneous rearrangements is so low as to indicate that the spontaneous presence of two independent breaks in the same nucleus is an event of extreme rarity. In irradiation experiments we can, of course, produce large numbers, not only of 2-break rearrangements, but also of the more complex types involving 3, 4, 5 or more breaks. The most complex rearrangement ever obtained was one in *Drosophila melanogaster* which, according to Kaufmann⁹⁷, involved at least 32 breaks and possibly more.

When an organism is subjected to irradiation with ionizing radiations (α -, β -, and γ -radiations, X-rays, protons, or neutrons) various effects are produced in its nuclei. There may be a temporary inhibition of mitosis, i.e. nuclei in the resting stage or early prophase which would otherwise have divided may be prevented from doing so. Chromosomes which were in late prophase or metaphase at the time of irradiation may undergo quite general alterations in the surface-properties, causing them to stick together or to undergo other changes (Alberti and Politzer^{2,3}; White²¹⁸; Marquardt¹²⁵). Finally, a certain number of chromosome- or chromatid-breaks will be produced. The actual number of breaks produced in any particular material by a given ionization dosage is almost impossible to determine, since the majority of breaks undergo restitution within the first few minutes and hence cannot be detected. Lea and Catcheside¹¹¹ have calculated by indirect methods that the number of 'primary breaks' produced in *Drosophila* per 1,000 *r*-units is about 0.75 per sperm. In these *Drosophila* sperms it appears that all the primary breaks remain 'open' until the time of fertilization, so that it makes no difference whether the irradiation is given at a high intensity for a short while or at a lower intensity over a longer period, the number of structural rearrangements produced depending solely on the total number of breaks which have accumulated during the life of the sperm (Muller¹⁴⁵). In *Tradescantia* pollen-grains, however, if the dosage is applied at a high intensity for a short time, so that all the primary breaks are coexistent, the yield of rearrangements is proportional to the square of the dosage, while if the irradiation is spread over a longer time, or fractionated, a certain amount of restitution takes place in the course of the experiment, so that the number of rearrangements produced is proportional to a lower power of the dose (Sax^{173,174}; Giles⁷⁵). At least, this is so for X-rays; in the case of neutrons and α -rays the number of rearrangements obtained is directly proportional to the dosage and independent of the intensity factor (Giles⁷⁵; Thoday²¹¹), thus suggesting that in most cases the two breaks involved in a rearrangement are produced by the same ionizing particle (Catcheside⁴⁵).

Most of the authors who have studied the effects of irradiation at different parts of the mitotic or meiotic cycle agree that metaphase is

the stage most sensitive to X-rays. This is the conclusion of Metz and Bozeman¹³⁵ and Reynolds¹⁶⁸ for *Sciara*, Sparrow¹⁹⁷ for *Trillium*, and Whiting²²⁷ for the eggs of the wasp *Habrobracon*. On the other hand, Darlington has claimed that the breakability of the chromosomes is greatest at the end of the resting stage and that prophase and metaphase chromosomes are 'protected by their coat of nucleic acid' from the effects of irradiation (Darlington and La Cour⁵⁷; Darlington⁵⁴). In *Tradescantia* pollen-grains grown on an artificial medium, Swanson^{207,208} found that the maximum yield of structural changes was obtained when the cells were irradiated in mid-prophase.

The effect of temperature during the experimental irradiation has been studied by various workers. As far as mutations are concerned, Timofeeff-Ressovsky and Zimmer²¹³ could find no effect of temperature on the production of sex-linked lethals in *Drosophila*. On the other hand, the rate of spontaneous mutation is known to be increased by rise in temperature.

As far as structural rearrangements are concerned, irradiation at higher temperatures leads to a decrease in the number of breaks observed in *Tradescantia*, probably because restitution is more frequent at higher temperatures (Lea¹¹¹). In *Drosophila*, Muller¹⁴⁵ reported that the temperature at the time of irradiation had no effect on the number of translocations produced, but his conclusions are not supported by the recent work of Baker¹⁰.

Treatment with ultraviolet radiation following X-rays leads to a decrease in the number of rearrangements obtained in *Drosophila* (Kaufmann and Hollaender⁹⁹), presumably because the ultraviolet radiation favours restitution; that ultraviolet radiation alone does not lead to the production of chromosomal rearrangements in *Drosophila* was shown by Muller and Mackenzie¹⁴⁹ and Demerec *et al.*⁶⁰. Treating male flies with near-infra-red radiation ($\lambda = 10,000 \text{ \AA}$) for 1–3 days before irradiation with X-rays led to a significant increase in the number of rearrangements obtained (Kaufmann, Hollaender, and Gay¹⁰⁰): the action of the infra-red radiation in 'sensitizing' the chromosomes to X-radiation is not understood.

The discovery that various types of chemical substances are capable of producing chromosome-breaks as well as causing gene-mutations when introduced into the cell is a relatively recent one (Auerbach^{7,8}; Auerbach, Robson, and Carr⁹; Darlington and Koller⁵⁵). The substances in question include mustard gas ($\beta\beta'$ -dichloroethyl sulphide), nitrogen mustards ($\text{CH}_3\cdot\text{N}(\text{CH}_2\cdot\text{CH}_2\text{Cl})_2$ and $\text{N}(\text{CH}_2\cdot\text{CH}_2\text{Cl})_3$). Allyl isothiocyanate, which has a weak capacity to produce mutations, has not been shown to be capable of causing chromosome-breaks (Auerbach and Robson⁹). Of the many carcinogenic substances found by Demerec⁵⁹ to possess mutagenic activity, only two (1, 2, 5, 6-dibenzanthracene and

benzpyrene) have been shown to produce chromosome rearrangements in *Drosophila*.

It is not known what causes spontaneous structural rearrangements. Obviously, the vast majority of these will be relatively simple in type (i.e. two-break rearrangements) since the probability of more than two breaks coexisting in the same nucleus is extremely small. Spontaneous rearrangements lead to (1) a situation in which several alternative gene-sequences are present in a local population or in the species as a whole, (2) permanent evolutionary changes in chromosome morphology. Thus in many species of *Drosophila* the natural populations are highly polymorphic in regard to gene-sequence, due to chromosomal inversions which are present in some individuals but not in others. All the differences in chromosome number and shape between different species, other than those due to polyploidy or reduplication of whole chromosomes, must be due to spontaneous inversions, translocations, duplications, and deletions which have occurred in the past. In the case of mutations it has been shown that their rate of natural occurrence is greater than could be accounted for if it were entirely due to cosmic radiation or to radioactive substances in the earth's crust. As far as spontaneous rearrangements are concerned, such a calculation has not been made, since their rate of occurrence is too low to be measured accurately.

i. Position Effects

Structural rearrangements are frequently accompanied by genetic effects, and these are of several kinds. In the first place, Catcheside and Lea have developed the theory that most, if not all, of the 'recessive lethals' produced by irradiation which are not due to demonstrable structural rearrangements, such as deletions or duplications visible in the salivary gland chromosomes, are the result of breaks which have 'restituted', i.e. where the two broken ends have joined up in the same sequence as before (see Lea and Catcheside¹¹²; Lea¹¹¹). Thus it seems that the process of breaking may inflict an 'injury' on a gene, even though the break joins up again.

In the second place, it is now well known that structural rearrangements are often accompanied by apparent mutation near the point of breakage. It has recently become clear that these are true 'position effects', due to a gene having an altered function when it is removed to a new situation in the chromosome. If the gene is returned to its previous position in the sequence (e.g. by crossing-over) it regains its original functions, thereby proving that it has not been permanently altered.

Position effects are of several types; some are merely changes in the degree of dominance of a gene, while others simulate gene mutations of various kinds.

So far, position effects are only known with certainty in *Drosophila* and in the plant *Oenothera*, where a single case has been reported by Catcheside⁴⁴. In maize, in spite of the relatively large number of structural rearrangements which have been studied, no position effects are known.

It is not always the genes next to the point of rearrangement which show the position effect—such effects may also be shown by genes located some distance from the site of union (in one case separated from it by 50 bands in the salivary gland chromosome). Probably some genes are more 'sensitive' to rearrangement than others, so that when chromosomes are broken in the immediate neighbourhood of 'insensitive' genes, it will be ones farther away that will show position effects most clearly. Rearrangements where one break is located in a heterochromatic region and one in a euchromatic region seem to be particularly liable to lead to position effects. A number of such rearrangements lead to 'mosaicism', i.e. they seem to produce an instability in a gene situated near the point of union, which henceforth undergoes frequent mutations in some of the somatic cells.

The mechanism of position effects is still unknown. On the one hand it has been suggested that it is due to some sort of local chemical interaction between neighbouring genes or between their products. This seems the most likely explanation, but the nature of the interaction can only be guessed at. On the other hand, Ephrussi and Sutton⁶⁵ have put forward the hypothesis that structural rearrangements may lead to disturbances in somatic pairing and that these may produce position effects. This would explain why position effects are known in *Drosophila*, whose chromosomes, in common with those of most Diptera, show somatic pairing, but not in maize, cotton, mice, and other organisms. But on Ephrussi and Sutton's hypothesis one would expect position effects to be much weaker or absent altogether when the rearrangement is present in the homozygous condition, which is emphatically not the case. A more probable hypothesis of the same general type would be that position effects arise when, in a structural rearrangement, two ends with different diameters are joined together (i.e. when one break is in a thick region, the other in a thin one).

j. Meiosis, Synapsis and Crossing-over

It is a striking fact that the meiosis of plants and animals, lilies and grasshoppers, protozoa and fungi, seems to follow the same general course, the details being usually very similar in the two sexes, as far as the chromosomes are concerned. There are, however, a few groups of animals, such as the scale-insects and lice, in which the meiosis of the males is of a highly modified type, and the gall-midges, where meiosis is anomalous in both sexes. A less profound modification of the

meiotic process occurs in the higher Diptera (including *Drosophila*) in which crossing-over has been abolished in the male. These exceptions, however, merely emphasize the general uniformity of meiosis in most groups of organisms.

Essentially, meiosis consists of two successive divisions in the course of which the chromosomes only split once. Two features of the process claim special attention—the pairing (synapsis) of homologous chromosomes, and the breakage and reciprocal re-fusion of the chromosomes which constitutes crossing-over, and leads to the appearance of *chiasmata* (X-like connexions between the two chromosomes of which each ‘bivalent’ is made up).

Synapsis occurs at an early stage (‘zygotene’) in the prophase of the first meiotic division. Usually the chromosomes are ‘polarized’ before pairing actually starts; that is to say, all their ends are brought together on one side of the nucleus. The actual process of pairing seems to be due to a highly specific force of attraction between homologous genes or chromomeres, since even when structural rearrangements such as inversions or translocations of quite short portions are present in the heterozygous condition, they nevertheless usually manage to ‘find’ their homologues and pair with them.

In general, pairing seems to be a two-by-two attraction (Darlington⁵⁰ and later papers). That is to say, in a triploid individual, where each chromosome is present in triplicate, A_1 may pair with A_2 in one region and with A_3 in another, but all three do not come together in the same part of their length. Furthermore, the homologous chromosomes in a nucleus do not behave as if one was emitting a negative pairing force and the other a positive pairing force; that is to say, any chromosome region can, and normally will, pair with any other homologous region.

It will be obvious that there are no inorganic analogies for a system of perhaps 10,000 different forces of attraction, each capable of being exerted over a distance of several μ and being sufficiently powerful to bring the threads together from that distance, through a somewhat viscous medium. Fabergé⁶⁶ has considered some of the physical properties which such forces must possess: his particular suggestion, namely, that pairing depends on some form of Bjerknes effect (homologous genes acting as pulsators with a characteristic frequency and in phase with one another) may prove to be wrong; but there can be no doubt that he has focused attention on the need for consideration of chromosome pairing by physicists. Most authors have assumed that chromosome pairing involves an attraction between like molecules, but Friedrich-Freksa⁶⁷ has put forward a hypothesis according to which each gene consists of two parts, the nucleic-acid part of each attracting the protein part of the homologous gene and/or vice versa. Possibly the explanation of the mechanism of chromosomal synapsis will come from work

on analogous phenomena in suspensions of virus particles (Bernal and Fankuchen³⁰), as suggested by Muller¹⁴⁸.

If synapsis is the first mystery of the meiotic process, crossing-over (chiasma-formation) is the second. Ordinarily, at meiosis each bivalent acquires at least one cross-over and frequently several. At a stage when the bivalent (composed of two homologous chromosomes paired throughout their length) is effectively 4-stranded (regardless of how it actually appears under the microscope), two of the strands, *one derived from one chromosome and one from the other*, break at precisely the same level. They then proceed to rejoin in the opposite way to the original one. The result of this is that when the pairing-attraction between the chromatids of maternal and paternal origin comes to an end (as it usually does shortly after crossing-over has taken place) the two halves of the bivalent are still held together at the points of crossing-over, which now appear as the chiasmata of the cytologist.

There are a number of aspects to the process of crossing-over which require further elucidation. In the first place, what causes the threads to break? Secondly, why do two always break at exactly corresponding levels? Thirdly, why, having broken, do they rejoin 'the other way round'?

With regard to the first point, Darlington⁵¹ has suggested that breakage results from a tension imposed on the threads by a relational torsion between the homologous chromosomes. When the chromosome splits (Darlington assumes that this takes place at pachytene, but the point is really immaterial, since the 'effective' split may occur later than the duplication process) the chromatids are unable to withstand the strain and accordingly break, allowing a relaxation of the torsional strain to take place. But it is very dubious whether such a relational torsion is always present, and in very minute chromosomes, no longer than their own diameter, it is difficult to see how it could be present. Nevertheless, such chromosomes, if regular members of the chromosome complement of the species, always undergo crossing-over at meiosis. Moreover, in *Neurospora* the observations of McClintock¹²¹ demonstrate that the two chromosomes at pachytene lie strictly parallel 'like railroad tracks'.

In ring-X chromosomes of *Drosophila*, too, the genetical evidence shows that crossing-over is almost completely normal, although such chromosomes can hardly be relationally coiled about one another in a bivalent composed of two rings. The behaviour of these ring-X chromosomes at meiosis also demonstrates that sister-strand crossing-over (i.e. crossing-over between the two chromatids derived from the same chromosome) does not occur.

An entirely different theory of crossing-over was put forward by Belling²⁷, who assumed that the 'new' interchromomeric connexions only

develop after the chromomeres have divided. The theory is somewhat complex and does not appear to have received the recognition it deserves. But in view of certain difficulties in the way of accepting either Belling's or Darlington's theory of crossing-over it seems more probable that the phenomenon is due to an alternation of different types of segments in the chromosome in which the reproduction and splitting of the nucleo-protein threads is not quite synchronous. If the two homologues are held together by a pairing attraction so long as they are effectively unsplit and repelled as soon as they are effectively split, then a tension is bound to develop where a split region meets an unsplit one (White²²¹). This would account for the known fact that in many grasshoppers the chiasmata are localized in the neighbourhood of 'frontiers' between euchromatic and heterochromatic regions.

One feature of crossing-over that needs to be emphasized is that at least one chiasma is formed in each normal bivalent. This, in itself, renders it unlikely that the phenomenon is caused by chance overlaps or relational coiling which, even if they occur, are likely to be highly variable from one cell to another.

iv. THE NATURE OF THE GENE

Many attempts have been made to arrive at estimates of the number and size of the genes. In the first place, we may consider those which depend on the direct evidence of microscopical observation. The salivary gland X-chromosome of *Drosophila melanogaster* contains about 1,000 bands, which have actually been mapped, and it is probable that each of these corresponds to a gene (see page 213). On the other hand, it may contain more bands, and hence more genes. Muller and Prokofieva's¹⁵⁰ very careful genetical and cytological mapping of a particular region suggested that the genes are, on the average, about 0.125μ apart, so that there may be as many as 1,600 (rather than 1,000) in the 200μ -long salivary gland X-chromosome. Now the volume of the euchromatic (i.e. genetically active) part of this chromosome at a spermatogonial metaphase is about $0.34\mu^3$. Bearing in mind that this consists of two chromatids and assuming the chromosome to be entirely composed of genes, this would give each gene a volume of about $100,000\text{ m}\mu^3$, corresponding to a cube whose edges are $46\text{ m}\mu$ long. This is clearly a maximum size for the gene, since it not only assumes that there are no non-genic portions of the chromosome (matrix, intergenic connexions), but also that each chromatid is a single string of genes and is not divided into half-chromatids, quarter-chromatids, &c. These assumptions are unlikely, particularly when we consider that the volume of the chromosome set of a grasshopper is perhaps 100 times that of the chromosome set of *D. melanogaster*; it is inherently improbable that grasshoppers have

100 times as many genes as *Drosophila*, or that their genes are 100 times the size of those of *Drosophila*.

Using entirely different premisses, and basing their calculations on the 'target theory' of the biological effects of radiations, which assumes that ionization inside a gene invariably causes a mutation and that ionization outside a gene never does, Lea and Catchside¹¹² arrived at an estimate of 4–8 m μ for the diameter of the gene. They believe this estimate to be correct to a factor of 2. The diameter of the gene thus probably lies between 4 and 46 m μ , and the total number of genes in the haploid chromosome set of *Drosophila* is perhaps between 5,000 and 10,000 (see Section g). They are almost certainly nucleo-protein in nature, but whether each gene contains two types of nucleic acid and two types of protein or only one of each, as seems more probable, is still unknown.

The most recent work of Tatum and Lederberg²¹⁰ and Lederberg¹¹³ indicates that heredity in bacteria also depends on particulate genes and that in one species of bacterium at any rate (*Escherichia coli*) these genes undergo a form of re-combination remarkably like crossing-over in the higher organisms. As far as the bacterial viruses (bacteriophages) are concerned, the work of Luria¹¹⁷ suggests that they are composed of, or contain, a number of genetic sub-units, perhaps as many as fifty in some cases.

Probably classical genetics and cytology have gone about as far as they are capable in exploring the gene: it remains for the chemists to complete the analysis of what Beadle²³ has called the 'irreducible units of living systems'.

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FIGS. 1 and 2. Fifth cleavage division in the mite *Pediculopsis graminum* showing the elimination-bodies between the chromosomes. Fig. 1 is an early stage of anaphase, Fig. 2 a slightly later one. Preparation by Dr. K. W. Cooper. Fixation: Navashin's fluid. Staining: Heidenhain's haematoxylin

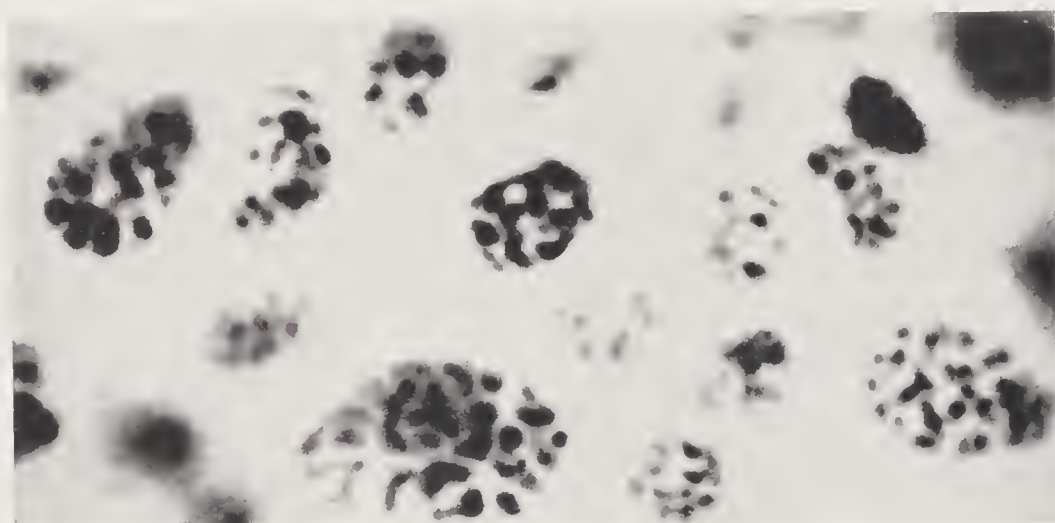


FIG. 3. Diploid, tetraploid, octoploid and (?) 16-ploid somatic nuclei in the wall of the testis of the locust *Schistocerca gregaria*. Fixation: Flemming's fluid. Staining: Gentian violet by Newton's method

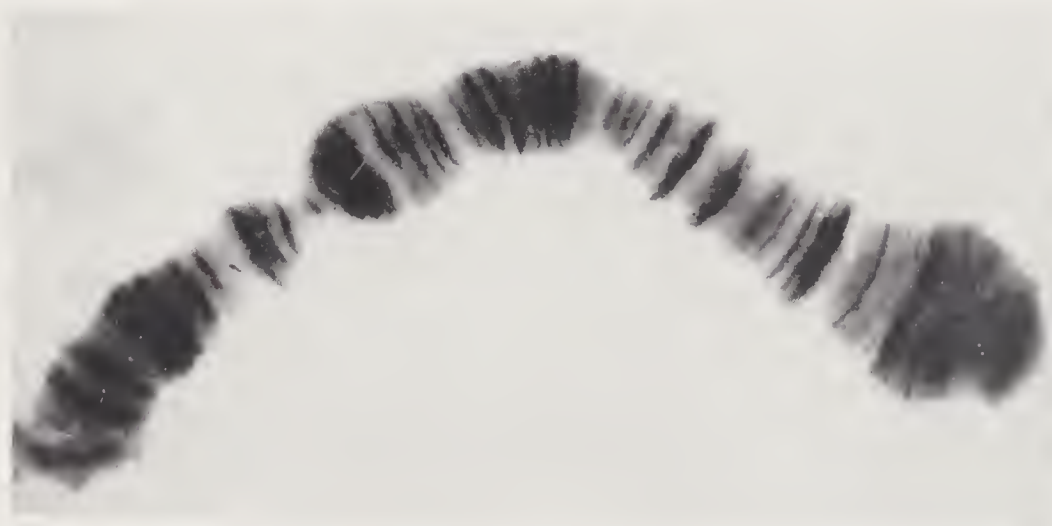


FIG. 4. Part of a salivary gland chromosome in *Chironomus* sp. Fixation and staining: 1 per cent. orcein in 45 per cent. acetic acid



FIG. 1. X-chromosome, showing heteropycnosis and spiral structure in a spermatogonial resting nucleus of the long-horned grasshopper *Platycleis grisea*. *Fixation*: Strong Fleming's fluid. *Staining*: Gentian violet by Newton's method

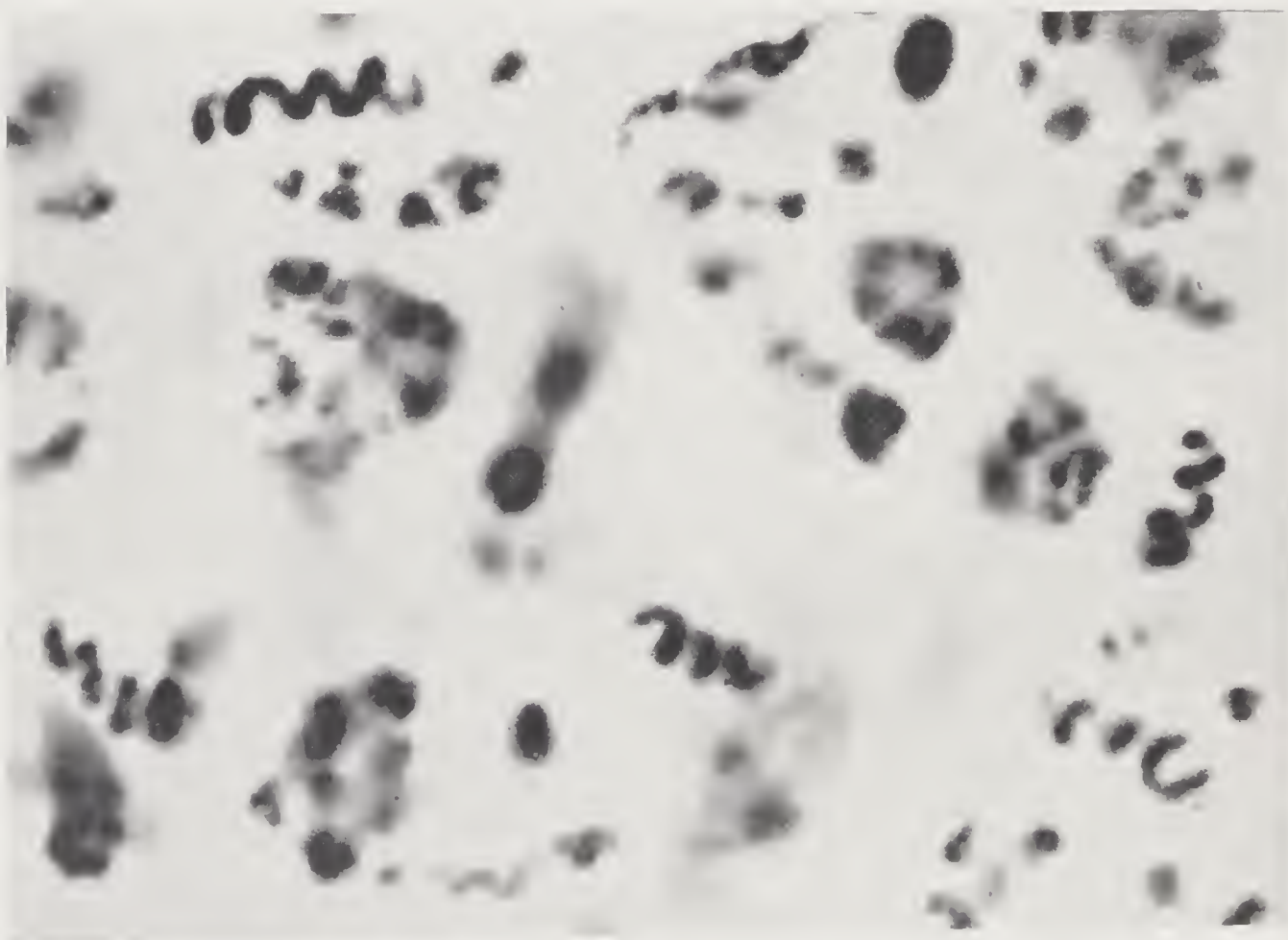
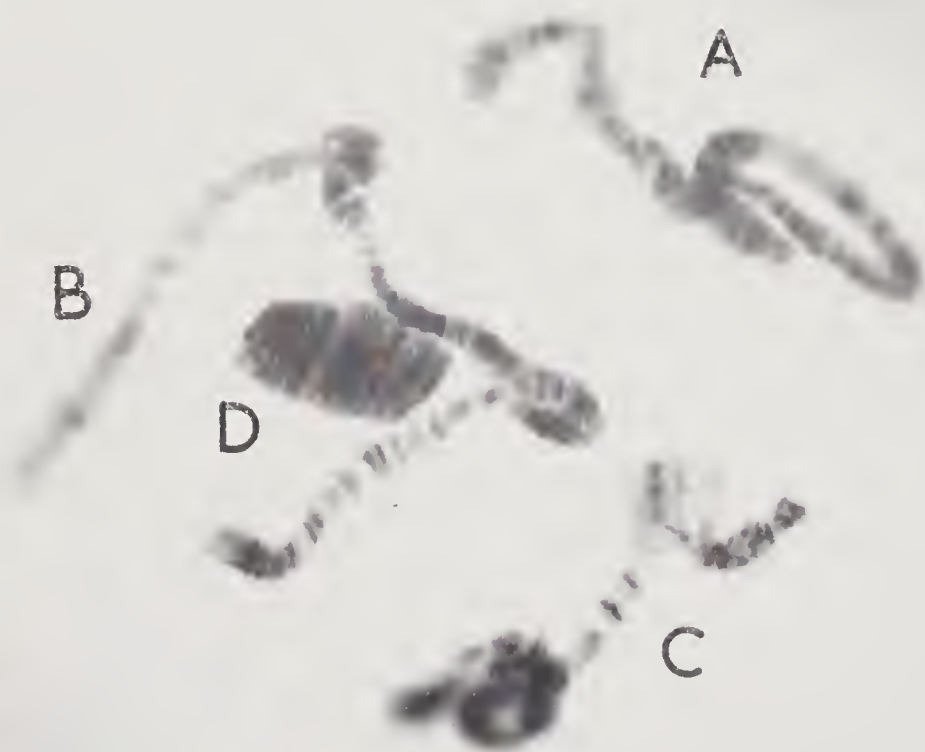


FIG. 2. X-chromosomes showing spiral structure in spermatogonia of the long-horned grasshopper *Insara gracillima*. *Fixation*: San Felice's fluid. *Staining*: Gentian violet by Newton's method



1



2

Chromosomes of *Camptomya* sp.



FIG. 1. Chromosomes of *Lestodiplosis* sp. 1

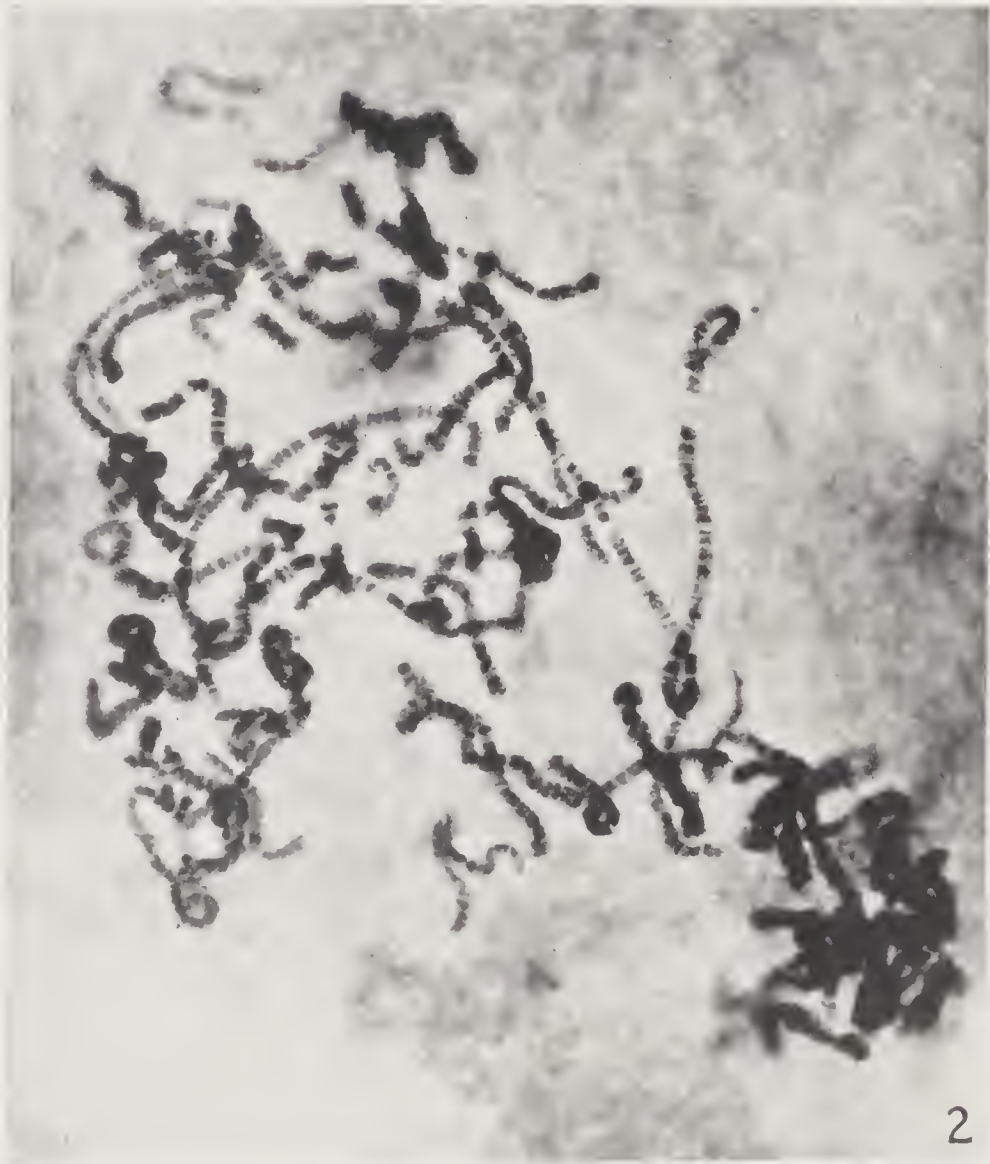


FIG. 2. Chromosomes in a giant cell nucleus of *Lestodiplosis* sp. 1

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DESCRIPTIONS OF PLATES

PLATE 1

FIGS. 1 and 2. Fifth cleavage division in the mite *Pediculopsis graminum*, showing the elimination-bodies between the chromosomes. Fig. 1 is an early stage of anaphase, Fig. 2 a slightly later one. Preparation by Dr. K. W. Cooper. *Fixation*: Navashin's fluid. *Staining*: Heidenhain's haematoxylin.

FIG. 3. Diploid, tetraploid, octoploid, and (?)16-ploid somatic nuclei in the testis wall of the locust *Schistocerca gregaria*. *Fixation*: Flemming's fluid. *Staining*: Gentian violet by Newton's method.

FIG. 4. Part of a salivary gland chromosome of *Chironomus* sp. *Fixation and Staining*: Aceto-orcein.

PLATE 2

FIG. 1. X-chromosome, showing heteropycnosis and spiral structure, in a spermatogonial resting nucleus of the long-horned grasshopper, *Platycleis grisea*. *Fixation*: Flemming's fluid. *Staining*: Gentian violet by Newton's method.

FIG. 2. X-chromosomes showing spiral structure in spermatogonia of the long-horned grasshopper, *Insara gracillima*. *Fixation*: San Felice's fluid. *Staining*: Gentian violet by Newton's method.

PLATE 3

FIGS. 1 and 2. *Camptomya* sp. salivary gland nuclei moderately flattened. C. Nucleolar chromosome. D. The wide chromosome. *Fixation and Staining*: Aceto-orcein.

PLATE 4

FIG. 1. The chromosomes of ordinary salivary gland nuclei in *Lestodiplosis* sp. 1. From an individual heterozygous for a subterminal inversion in Chromosome A. *Fixation and Staining*: Aceto-orcein.

FIG. 2. Photomicrograph (taken with a Leitz 4 mm. dry apochromatic objective) showing the chromosomes in a giant cell nucleus of *Lestodiplosis* sp. 1, well spread out. This nucleus is structurally homozygous (i.e. without inversion). *Fixation and Staining*: Aceto-orcein.

CHAPTER VI

MITOCHONDRIA AND THE GOLGI COMPLEX

By G. H. BOURNE

i. INTRODUCTION

NO cellular components have aroused more controversy than the mitochondria and the Golgi apparatus and, as a result, any attempt to discuss them is fraught with pitfalls. Since the earlier work on these organelles has been adequately reviewed in 1924 by Cowdry⁶¹ and in 1931 by MacBride and Hewer¹⁵⁴, reference is made in this chapter mainly to work which has been published since the appearance of these reviews. In the case of the Golgi apparatus there are even more recent summaries of our knowledge; one by Kirkman and Severinghaus in 1938¹³⁸, one by Hirsch in 1939¹⁰⁹, and one by Hibbard in 1945¹⁰⁷. In consequence only certain aspects of the study of the Golgi apparatus will be dealt with in this chapter. Those who wish for further enlightenment on the problem of neutral red cytology, the role of the Golgi apparatus in male and female germ cells, and other problems not dealt with here should consult these reviews.

ii. MITOCHONDRIA

a. General

Mitochondria or chondriosomes are elements of definite form in the cytoplasm of all cells. They lie freely in the cytoplasm, possess the power of independent movement, and may take the form of filaments, of rods, or of granules. Although they were originally described by Altmann⁵ between 1880 and 1890 and were called Altmann's granules, they had, according to Cowdry⁶⁰, been seen and described some years before.

Following the description of these granules there was a period in which various workers showed that many structures thought to be characteristic of cells, including Altmann's granules, could be reproduced at will by the use of different fixatives and staining procedures in models of cells. The result was that Altmann's granule theory was discarded. In 1897, however, Benda demonstrated similar objects in cells without realizing that they were the same as those described by Altmann. He called them 'mitochondria' and was able to demonstrate them in both fixed and living cells.

There are now something like fifty synonymous terms in existence for these structures. The term 'mitochondria', which has remained in common use, comes from two Greek words meaning respectively 'thread' and 'granule'. The name is apt because mitochondria are either in the

form of filaments or small rods and granules; in the cells of some organs the latter form is typical. In most animal cells the shape of the mitochondria is characteristic for one particular organ. For example, in the adrenal cortex they are in the form of short rods or granules, whereas in the cells of the intestine they are more usually filamentous. They may change in form in secreting glands. Thus in the pancreas they are believed to break up and form granules which eventually metamorphose into zymogen granules. The diameter of mitochondria in individual cells is extraordinarily constant, and though they may increase in size they never do so by increasing their girth, but by an increase of length. Sometimes mitochondria may clump together and coalesce; the bodies so formed are called chondriospheres. Such bodies often occur in scurvy.



TEXT-FIG. 1. Mitochondria in scurvy. Re-drawn after Bourne²⁷.
A. Liver cell; B and C. Kidney cells.

Mitochondria can be seen quite clearly in tissue-culture cells under dark ground illumination. Here they appear in a state of constant movement. This movement is of two types: a transposition of the whole mitochondrion from one part of the cell to another (due probably to varying electric charges on the mitochondrial and various cell membranes), and a wriggling movement by the mitochondrion itself. In addition, filaments may break up into rods and these may disintegrate further into granules. Granules and rods may join up again and form filaments, and the filaments may form temporary networks. Lewis has seen mitochondria in a living cell making a series of journeys from the nucleus to the cell membrane and back again. Mitochondria are frequently clumped around the archoplasmic part of the cell.

According to Policard and Mangenot (quoted by Bensley and Gersh²⁴), mitochondria are sensitive to heat and appear to 'melt' suddenly at temperatures of 48° to 50° C., but Bensley and Gersh themselves found that heating frozen dried tissues to 140° C. for several hours *in vacuo* had no effect on the mitochondria.

Jefferson¹²⁸ in 1945, using blow-fly larvae, found that even in living tissues mitochondria were sensitive to heat, and suggests that the liberation of mitochondrial lipoids by heat might result in an upsetting of the enzyme systems of an animal and lead to irreversible heat injury. There is evidence that high temperatures can cause the disintegration of mitochondria in living cells in plants, frogs, fish, guinea-pigs, and rabbits (Belehradek²⁰; MacCardle¹⁵⁵).

Mitochondria seem to be of universal occurrence in living cells. They are well developed even in amoeba. Only in bacteria is their presence doubtful. Wallin²²² and others have compared mitochondria to intracellular symbiotic bacteria, but this view is not supported by the majority of cytologists.

Mitochondria are very sensitive indicators of cellular injury. Crushing of tissues with the forceps before fixation is liable to cause them to break up into granules. Mitochondria do not normally stain with osmium tetroxide (osmic acid) but in injured cells they frequently do, and this may indicate an increase in the amount of unsaturated fat or lipoid. In scurvy the mitochondria break up and coalesce (Text-fig. 1, A, B, and C), in beri-beri they do not change. In cyanide poisoning mitochondria maintain their shape, but their movement is inhibited. It may be of interest that mitochondria have been regarded as possessing a respiratory function, and cyanide is known to inhibit certain respiratory enzyme systems.

In the formation of fat and lipoid in the animal cell the number of mitochondria decreases. In plant cells it decreases with the formation of plastids. It is claimed by some workers that plastids are formed from the Golgi apparatus, but Guillermond⁹⁹ has provided fairly convincing evidence of their origin from mitochondria. In primitive red blood cells the mitochondria decrease with the appearance of haemoglobin. Various authors have claimed that they are associated with a wide variety of cellular products.

Mitochondria are believed by some to play a part in the formation of yolk in some eggs and to form the middle piece of the mature spermatozoön. (For a detailed review of this subject see MacBride and Hower¹⁵⁴.) Meves¹⁶⁶ has observed a mingling of male and female mitochondria after fertilization in *Ascaris*, and believes that a conjugation between them takes place. In the developing embryo of the bat the male mitochondria sometimes pass to only one of the first two cells formed by division of the fertilized egg. In some Echinids they have been traced into only one of the cells of a 32-celled embryo. In other cases, e.g. *Nereis*, however, the middle piece of the sperm, which carries the mitochondrial material, does not enter the egg at all.

The measurement of the amount of mitochondria in cells presents difficulties owing to the unavoidable differences in the techniques and the impossibility of complete standardization. Nevertheless, Thurlow²¹² has made an attempt to do so in the nerve cells of white mice. She found that there was a constant number of mitochondria per unit volume of cytoplasm. The cells of some organs, however, may be so different in mitochondrial content from those of other organs that the difference may be seen by simple microscopical observation.

Mitochondria tend to aggregate around the spindle in cell division.

and as a result of the pinching-in of the cytoplasm they are divided between the two cells in approximately equal quantities. In the case of fibroblasts the mitochondria break up into smaller bodies and become scattered through the body of the cell. Many, however, remain filamentous. Division of the cell body again results in approximately equal distribution (Plate 1, Fig. 2).

Pollister¹⁸¹ has shown that mitochondria lie parallel to the course of diffusion in cells. The orientation of the mitochondria in cells is due to the 'parallel orientation by long protein molecules—the structure proteins—of the hyaloplasm'.

b. Structure and Composition of Mitochondria

That mitochondria are cell organs of definite shape and structure was demonstrated by Beams and King^{18,19}, who found that in the ultra-centrifuged cells of rat liver the mitochondria maintained their shape and were thrown centrifugally within the cell. The latter fact indicates that they are denser than the cytoplasm.

There is good evidence that mitochondria have a double structure. It may be seen sometimes in mitochondria *in vitro*, and in any case it may become more obvious in large masses of mitochondrial material. The outer sheath or cortex of this double structure from the operation of the Gibbs law probably contains a large amount of fat or lipid. Mitochondria are stained by a method similar to that used for staining *Mycobacterium tuberculosis* and *Mycobacterium leprae*. These bacteria are stained by treating them for some minutes with a hot phenolic solution of basic fuchsin. Once stained in this way they resist the destaining action of acid alcohol. They are known as 'acid-fast' bacteria, and their staining idiosyncrasies are believed to be due to the presence of a waxy or lipoidal coat. Mitochondria are stained with hot acid fuchsin and resist the decolorizing action of picric acid for a longer period than most of the other cellular constituents. (For details see Cain^{43,44}.) Up to the time of the development of the freezing-drying method for histological fixation it was believed that most of the substance of mitochondria was soluble in acetic acid, alcohol, ether, chloroform, acetone, and other fat solvents. Mitochondria have a low refractive index which supports the theory of their lipid nature, and on occasions they may stain with osmium tetroxide. According to Cowdry their smooth outlines suggest myelin bodies.

Baker¹² has applied his acid haematin test for lipins to a variety of tissues and finds that in each case the mitochondria give a positive reaction. The tissues he used included convoluted tubules of frog and mouse kidney, mouse liver cells, and mouse spermatozoa (the middle piece of which is of mitochondrial origin).

Examination of isolated mitochondria under the electron microscope

(Claude and Fullam⁵⁵) shows that some mitochondria contain small bodies up to 100 m μ in diameter which have a greater electron density than the rest of the mitochondria. Mitochondria of spermatozoa also show internal bodies (Schmitt¹⁹⁶).

It seems likely, therefore, that lipin is a characteristic constituent of mitochondria, and further information on the subject has been provided by the work of Bensley and Gersh²⁴, using the freezing-drying method for fixation of tissues. This method was originally introduced by Altmann, who froze tissue at a temperature of -15° to -20° C. and then dehydrated it in a vacuum. This technique was subsequently improved by Gersh⁹⁰. As outlined by Bensley and Gersh²⁴ the new technique is as follows. The tissue on removal from the body is frozen immediately in liquid air. It is then placed in a chamber cooled to -20° C., and with the aid of a 'Hy-vac' pump and a mercury vapour pump an initial vacuum of 0.001 mm. of mercury is established, and as the tissue water evaporates the vacuum rises to 0.0001 mm. of mercury. This vacuum is maintained for 12 hours, by which time the drying of the tissues is presumed to be complete. They are then transferred directly into melted paraffin for a few minutes, then embedded, sectioned, and mounted without the use of water. The dehydrating effect of the vacuum depends, according to Gersh, upon the fact that water cooled to -20° C. has a vapour pressure of 0.9441 mm. of mercury. In a vacuum of 1×10^{-4} the vapour pressure of the ice in the tissue will be much greater than that of such a vacuum; the result will be a rapid evaporation of the water and dehydration of the tissues. The advantages of this technique are that the solid constituents of the cells are precipitated *in situ* without the extraction and diffusion which occurs as a result of the use of fluid fixatives, and they are precipitated chemically unchanged.

Bensley and Gersh found that the mitochondria of liver cells which had been treated by this technique were unaltered by the application of acetic acid, or by the extraction of the tissue for 50 hours in a Soxhlet apparatus with alcohol, chloroform, acetone, sulphuric ether, or petroleum ether. The same authors point out that it is possible that even these hot fat solvents were insufficient to 'break up a firm protein lipid combination'. Mitochondria have been shown to contain appreciable amounts of fatty material (see p. 242), and since they probably possess an outer adsorbed layer of protein, it is not unlikely that the change in physical state of this protein film, engendered by the freezing-drying process, would make it impermeable to fat solvents. That protein constitutes as well an important part of the 'body' of mitochondria is shown by the fact that these bodies disappear as a result of the activity of proteolytic enzymes (see also p. 240).

It is well known that mitochondria stain specifically with Janus

green B (diethylsafraninazodimethylalanin), although Hirsch¹⁰⁹ claims that this stain also demonstrates the pre-substance of the Golgi apparatus; it also stains certain bacteria. Cowdry⁶⁰ states that the action of Janus green B is due to the diethylsafranin part of the molecule because a dye of this composition also stains mitochondria specifically. In order to differentiate more clearly between mitochondria and bacteria, Horning¹¹⁷ has used a dye, produced by Robertson, which stains mitochondria alone. This dye is Janus red, and it is the sodium salt of diethylsafranin monocarboxylic acid.

Robertson¹⁹² observed that one drop of a saturated solution of safranin added to a solution of trypsin caused the formation of a coloured precipitate. Holtzberg¹¹⁶ later demonstrated the proteolytic activity of this precipitate. It was then shown by Marston¹⁵⁹ that other azine dyestuffs, including dimethyldiaminotoluazine hydrochloride (neutral red), were also able to precipitate proteolytically active trypsin from solution. When these dyestuffs were added to crude gland extracts the resultant precipitate showed only proteolytic and no lipolytic or diastatic activity. Azine dyestuffs will, however, precipitate a number of proteolytic enzymes, e.g. pepsin, trypsin, erepsin, and papain. The linkage of dye to enzyme takes place, according to Marston, through the basic nitrogen of the heterocyclic azine ring. In view of these results Marston pointed out that the reaction of mitochondria with Janus green B may indicate that the former contain proteolytic enzymes.

It is of interest that neutral red, which does not stain mitochondria, also precipitates proteolytic enzymes. Hirsch¹⁰⁹ points out that both neutral red and Janus green B stain what he describes as the 'pre-substance' of the Golgi apparatus. On the other hand, the true Golgi apparatus rarely stains with Janus green, although it may (or at least vacuoles in close association with it may) stain with neutral red. These differences in staining ability suggest—if they are dependent upon the presence of proteolytic enzymes—either that different groups of enzymes are present in the two organs, or that the attachment of the enzyme to, or that the nature of the substrate is different. It must be remembered, however, that proteolytic enzymes may be distributed in the body of the cytoplasm, and the impression that these enzymes are present in the mitochondria or pre-substance may be due to the fact that the chromophoric groups as they are formed become concentrated in these bodies.

Mitochondria gradually reduce Janus green B. Guillermond and Gautheret¹⁰⁰ point out that this is not a simple reduction. The colour of the dye changes from greenish-blue through red to colourless, and in the red condition the dye may diffuse out into the cytoplasm or into the nucleus or into both.

Ludford¹⁵⁰ found that after the application of a dilute solution of methylene blue to tissue cultures, the mitochondria of the cells stained

a brilliant blue. Cytoplasmic granules and vacuoles stained a fainter blue. In some cells after a time there was a diffuse staining of the cytoplasm, but the mitochondria still remained visible because of their more intense blue colour. Ludford found that KCN inhibited the vital staining of the mitochondria with methylene blue. Exposure of the cells to a bright light caused a rapid bleaching of the blue colour of the vitally stained mitochondria. Ludford believes that this may be due to injury of the cell organs which results from intense illumination. The disappearance of the colour may be due to the presence of reducing substances present in the mitochondria, or occurring in them as a result of injury, the action of which is catalysed by the bright light. It is known that reducing substances are present in tissues which reduce methylene blue under the stimulus of light; vitamin C, for example, is one of these substances.

Ludford also found that fewer of the mitochondria of dividing cells stained with the methylene blue than those of resting cells, and that in less healthy cells the number staining was likewise decreased. Leuco-methylene blue was found to stain the mitochondria very poorly or not at all, but *in vitro* staining was also obtained by the use of toluidine blue and of brilliant cresyl blue. Some staining of the mitochondria was also obtained with methylene blue by the use of the *intra vitam* and the *supra vital* methods, but there was considerable variation of staining by the various cells, and Ludford assumes that this is related to the different oxidation and reduction processes in them. Ludford concludes that 'the staining process is an expression of the vital activity of the cells'. He also points out that the ability to stain mitochondria vitally is not the prerogative of any one dyestuff, since while methylene blue is a thiazine dye and toluidine blue is structurally related to it, brilliant cresyl blue is an oxazine dye.

The discovery by Hogeboom *et al.*^{111,112} that the major proportion of the succinic dehydrogenase and an appreciable proportion of the cytochrome oxidase activities of the liver cell are present in the mitochondria suggests that these organelles, in the liver cells at least, are the major sites of aerobic respiration. It seems, therefore, that mitochondria might be regarded as important respiratory centres in the cell, provided, of course, that the succinic dehydrogenase really is in the mitochondria and is not merely adsorbed on to them from the homogenate. If succinic dehydrogenase exists in the mitochondria of the living cell, this may be due to the fact that it was adsorbed in the living cell on to a convenient surface. Perhaps it is also adsorbed on to the surface of the Golgi material, and if we could extract this material from the cell with the same facility with which we can extract mitochondria it might be possible to show the enzyme to be present in this organelle as well.

The presence of members of the vitamin B complex in mitochondria

(*vide infra*) is of interest in view of the fact that some of these vitamins are associated with, or form part of, the enzyme systems associated with the anaerobic metabolism of carbohydrates. The fact that these vitamins are present not only in the mitochondria but in other parts of the cell as well suggests that any participation by the mitochondria in anaerobic respiration is incidental, and that this process is a general function of the cell.

Kennedy and Lehninger^{134a} have shown that mitochondria in addition to possessing succinic dehydrogenase activity also catalyse the condensation of pyruvic acid with oxalo-acetic acid and the formation of succinic acid from α -keto glutaric acid. These three reactions represent steps of fundamental importance in the Krebs tri-carboxylic acid cycle.

Mitochondria also contain coenzyme I (of which nicotinamide is an important constituent) and cytochrome reductase, which is a flavo-protein. These two enzymes are a link between the Krebs tricarboxylic acid cycle and the cytochrome system. It appears, therefore, that mitochondria contain the whole enzymic equipment necessary for aerobic respiration of the cell. However, only about half the coenzyme I and cytochrome reductase activity of the cell is contained in the mitochondria, whereas these organelles contain 80 per cent. or more of the enzymes of the Krebs cycle and the cytochrome system, so it may be that the reaction between the mitochondria and the cytoplasm is necessary for aerobic respiration to proceed at its normal rate.

Pyridoxine and transaminase activity are also present in mitochondria, though only in the same concentration as in the rest of the cytoplasm. The fact that pyruvic, oxaloacetic, and α -keto glutaric acids are the corresponding α -keto acids of the amino acids alanine, aspartic, and glutamic acids, need only to be transaminated to form them, and are themselves formed as intermediary products in the course of the Krebs cycle, indicates the mechanism whereby mitochondria might synthesize fresh protein material, and so increase in size themselves, or synthesize protein for the cell as a whole. All the fatty-acid oxidase activity of the cell is also in the mitochondria, and so is 80 per cent. of the octanoxidase activity.

Joyet-Lavergne^{130,131} claims that mitochondria contain an oxidase system which oxidizes cobaltous salts to cobaltic, and that these stain the mitochondria green.

Mitochondria, although they appear to be of a fatty nature, do not stain with Sudan III; they may be expected to contain protein, but earlier workers found that they gave a negative reaction with Millon's reagent. Bensley and Gersh²⁴, however, believe that this may be due to the quality of the reagent used. They give the formula of a Millon's reagent suitable for use on cells (for details see their paper). Using this reagent Bensley has obtained positive results from the mitochondria

of many tissues. Frozen-dried sections were found to be particularly suitable for use with the reagent, and Bensley and Gersh obtained positive results with the mitochondria of the cells of *Amblystoma* liver prepared by this method.

The same authors found that in undenatured, frozen-dried sections of *Amblystoma* liver the mitochondria were destroyed by artificial gastric juice and artificial pancreatic juice (see results of Tarao²⁰⁹ on effect of proteolytic enzymes on Golgi apparatus). They appear to contain no iron or polysaccharides. Gatenby⁸⁷ has discovered that in *Limnaea* mitochondria are frequently coloured by a yellow pigment (probably a carotenoid). Extracted liver mitochondria also have a yellow appearance²². This suggested that small amounts of carotenoids, or perhaps of vitamin A, might occur in the mitochondria of cells of other animals. It was shown by Bourne²⁸ and Joyet-Lavergne¹³⁰ that, as a result of the application of antimony trichloride in chloroform solution, the liver cells of mammals showed the mitochondria as bright blue bodies. Joyet-Lavergne has applied this reagent to a wide variety of tissues and animals and claims that in every case the mitochondria show the same reaction. Antimony trichloride is known not to be specific for vitamin A but to give a greenish-blue colour also with carotenoid pigments; therefore, a blue reaction on the part of the mitochondria may indicate that they contain either vitamin A, or carotenoid pigments, or both.

Popper¹⁸³, using fluorescence microscopy, said that there were present in epithelial cells fine bars and droplets containing vitamin A which simulated mitochondria but which were not.

Jones¹²⁹, however, obtained precise localization of the Carr-Price blue reaction in individual mitochondria of haemoblastic cells. He made no attempt to differentiate between carotenoids and vitamin A.

Confirmation of the histochemical test for vitamin A in mitochondria (see Bourne²⁸ and Joyet-Lavergne^{130,131}) is given by Goerner^{93,94,95} who, after extracting mitochondria by Bensley and Hoerr's method, showed that 27–32 per cent. of their weight was made up of lipoids which contained vitamin A. He found that 100 mg. of mitochondrial lipoid contained 249–910 U.S.P. units of vitamin A. He stated that injection of dibenzanthracene decreased the vitamin A content of liver mitochondria but increased the total lipid content. He believed that under the influence of dibenzanthracene the liver appeared to lose its ability to split carotene into vitamin A. Goerner found also that the mitochondria of the cells of liver tumours, formed by injecting azo-toluene dyes into animals, contained no vitamin A, whereas those of the surrounding healthy cells contained the vitamin. Further, he found that injection of vitamin A increased the content of this vitamin in the mitochondria of healthy cells but not of tumour cells. An extension of this line of investigation would probably provide valuable results.

It has been shown by Leblond¹⁴², Bourne²⁸, and Giroud and his collaborators⁹² that mitochondria of some organs react with acetic acid/silver nitrate solution, which is believed to demonstrate vitamin C specifically in histological preparations. This was the case in some cells in the adrenal cortex, medulla (Plate 1, Fig. 4), corpus luteum, and anterior pituitary glands (Plate 1, Figs. 7 and 8) of mammals. In some cells the reaction appears in the Golgi apparatus and in others (more rarely) in both.

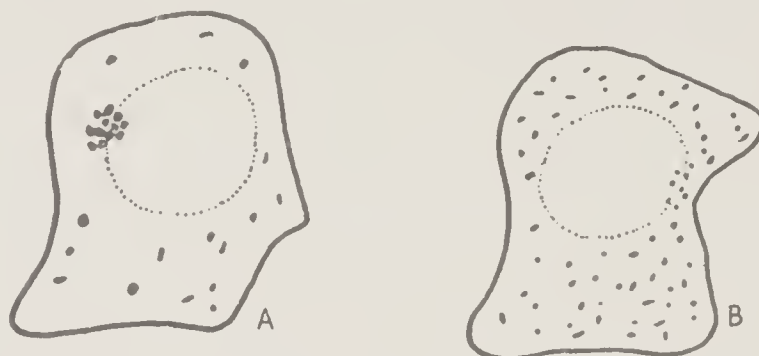
In addition to the possible presence of vitamin C, members of the vitamin B complex have also been shown to be present by Bensley²³ in mitochondria. Those recorded so far are vitamin B₁ (aneurin), riboflavin, nicotinic acid, pantothenic acid, and pyridoxin. However, the concentration of these vitamins in mitochondria was not shown to account for the major proportion of the amount of these vitamins present in liver homogenates. Thus the members of the B complex, though present in mitochondria, are not contained exclusively in these organelles. Bensley points out, however, that it is not impossible that the presence of these vitamins in the non-mitochondrial fraction of the homogenate may be due to the fact that they have been leached out of the mitochondria by saline solution used in the homogenization process.

It has also been found by Bourne²⁸ and Joyet-Lavergne¹³⁰ that mitochondria frequently give a positive nitroprusside reaction, suggesting that they contain glutathione or protein-bound SH (the latter is regarded as most likely by Giroud: personal communication). Joyet-Lavergne¹³¹ also claims that cadmium lactate reacts with cellular glutathione to form a cadmium glutathione compound which is visible under the microscope. In applying this reagent to a large variety of cells he found that his cadmium glutathione compound was localized, particularly in the nucleolus and mitochondria.

It has been shown by Bourne²⁷ that the Schultz reaction for cholesterol when applied to the adrenal gland showed the presence, in many of the cortical cells, of small granules and rods strikingly similar to mitochondria. A similar but less intense reaction was shown by the liver, and this mitochondrial reaction did not appear in any other organ treated with Schultz reagent. The absence of a reaction, however, may be due to the presence of glycerine, although this may be inhibited if stearic acid is also present (Whitehead²²⁷).

One of the most important advances in the study of the chemical nature of mitochondria has been made by Bensley and Hoerr²⁵, who extracted mitochondria from liver cells and subjected them to various chemical analyses. These two authors found that mitochondria of the liver of the guinea-pig and rabbit were completely insoluble in 0.85 per cent. saline, and that if a liver emulsion was subjected to differential centrifugation practically pure mitochondrial substance might be

obtained. Analyses of such extracted mitochondria showed that 43·6 per cent. of the dry weight was of a fatty nature. Cytologists have always regarded mitochondria as containing a large amount of lecithin or other phospholipid substances. Bensley and Hoerr, however, found that there was no precipitation of lecithin or cephalin when acetone was added to



TEXT-FIG. 2. Vitamin C preparations of A, cortical, B, medullary cells of rat adrenal, showing mitochondrial impregnation. In A, some granules may also be seen in the region of the Golgi apparatus.

a chloroform solution of mitochondria. The activation-of-cobra-venom/haemolysin test for lecithin was also negative. The test, however, indicated the presence of some free fatty acid. Dried mitochondria reduce osmium tetroxide to a brown colour and the separated fat does likewise; there is thus an unsaturated fatty substance present. Protein is also present in the mitochondria, and while no detailed study has yet been made of the protein fraction, preliminary investigation suggests the presence of two proteins with different isoelectric points. Subsequently Bensley²² published the following more accurate composition of dried liver mitochondria (figures approximated).

Proteins and unknowns	.	.	65 per cent.
Glycerides	.	.	29
Lecithin and cephalin	.	.	4 (some have more)
Cholesterol	.	.	2

Bensley²² believes that the surface of mitochondria is composed of a mosaic of protein and lipid micelles. He suggests that the main cortex of the mitochondrion is a mosaic of protein, glyceride, and cholesterol molecules. He believes that the failure of mitochondria to stain with Sudan III is due to the dispersal of the fatty substances. In general Bensley agrees with the suggested structure of mitochondria put forward by Bourne²⁸, except that he believes the cortex should contain both protein and lipoids* and not only fatty substances, as Bourne had suggested. The present author is in agreement with this amendment.

* The term 'lipoid' is used in a general sense to denote fats, phospholipids, sterols, &c.

The presence of cholesterol in Bensley's analysis is a possible confirmation of a histochemical observation (Bourne²⁸).

Bensley²³ has found a red pigment in the mitochondria and in the submicroscopic particles of the liver cell. This is present in smaller amounts in kidney mitochondria and still less in mitochondria of the testis cells. For various reasons he concludes that this pigment is derived from the oxidation of unsaturated fats, and particularly of phospholipins in the mitochondria. This leads him to suggest that 'in the liver cell the mitochondria and in particular the submicroscopic particles are the seat of highly active oxidative processes involving the metabolism of fats'.

Emmel^{77,78} found alkaline phosphatase activity associated with the mitochondria of some kidney tubule cells.

De Robertis^{69,70} has shown that in the amphibian hepatic cells the vesicular mitochondria may contain a pigment derived from haemoglobin.

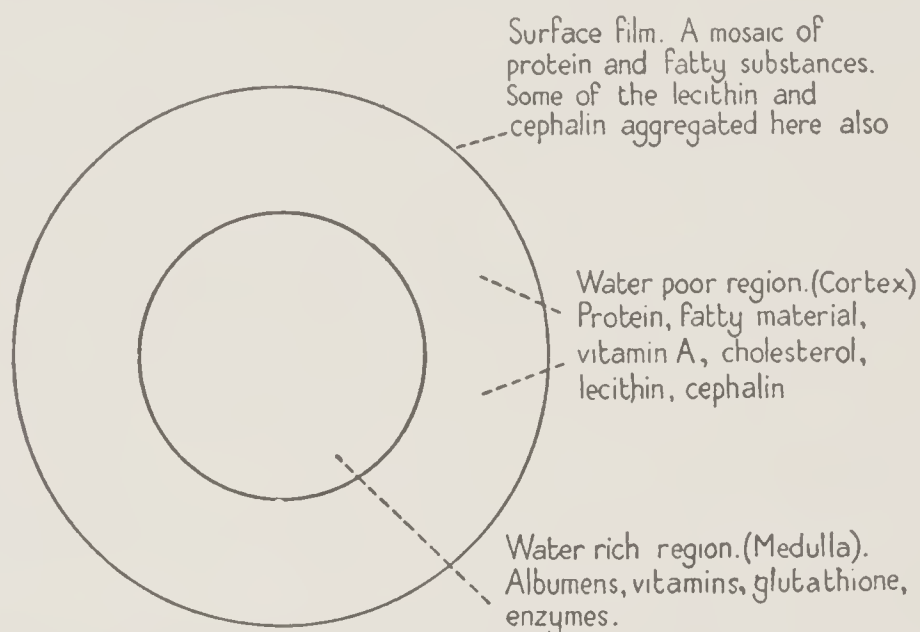
The following is a list of substances which are probably present in liver mitochondria. Some of these may occur in all mitochondria; the extent of the occurrence of the others is not known for certain.

- i. Protein.
- ii. Unsaturated (and probably saturated) fatty material. (Acids and glycerides.)
- iii. Lecithin and cephalin (small amount).
- iv. Cholesterol (small amount, probably larger amount in the mitochondria of adrenal cortical cells).
- v. Vitamin A+ /or carotene.
- vi. Vitamin C (probably only in certain endocrine mitochondria).
- vii. Members of the vitamin B complex (aneurin, nicotinic acid, riboflavin, pantothenic acid, and pyridoxin).
- viii. Glutathione or protein-bound SH. (probably in liver and some endocrine mitochondria).
- ix. Proteolytic enzymes.
- x. Cytochrome oxidase and cytochrome reductase.
- xi. Succinic dehydrogenase and other enzymes of the Krebs tri-carboxylic acid cycle.
- xii. Oxidase capable of oxidizing cobaltous to cobaltic salts.
- xiii. Ribonucleic acid.
- xiv. Transaminase.
- xv. Coenzyme I (containing nicotinamide).
- xvi. Fatty acid oxidase.
- xvii. Octanoxidase.

The water-soluble substances will probably be aggregated in the inner part of the mitochondrion, the fat-soluble substances in the cortex. Protein will probably be present in both parts. (See Text-fig. 3.) Seifritz has

shown, according to Frey-Wyssling⁸⁴, that protoplasmic movement has a pulsating rhythm, and the latter suggests that this may be due to periodic contractions of the polypeptide chains. It seems probable that the wriggling movement of mitochondria which can be seen in living tissue-culture cells is also due to the contraction of polypeptide chains of the proteins which are present in them.

Ludford (personal communication) has applied a number of the histochemical tests already mentioned to tissue-culture cells without obtaining any positive results for mitochondria. It has been found by Barnett



TEXT-FIG. 3. Possible distribution of various constituents of mitochondria.

and Bourne (unpublished work) that fibroblasts, except when degenerating and producing fat, show, when stained for vitamin C, only a very small or negative reaction. Furthermore, if vitamin C is added to the medium in which these cells are grown there is no increase in the ability of the cell to stain with the vitamin C reagent (Barnett, unpublished work). If, however, whole chick embryos are stained with the vitamin C reagent many of the fibroblasts give a quite good reaction. If a piece of rat intestine is incubated with vitamin C in normal saline, the connective tissue cells also take up appreciable amounts of the vitamin C. It appears, therefore, that the tissue-culture cell is in a different metabolic state from that of the same type of cell in organized tissue. Tests such as the nitroprusside, antimony trichloride, or Schultz do not give a positive reaction with the mitochondria of connective tissue cells, so that these tests, together with Ludford's observations, suggest that in these cells the mitochondria may not contain glutathione, vitamin A, or cholesterol.

c. *Function of Mitochondria*

That mitochondria are the respiratory centres of the cell has been a popular thesis with cytologists for some time. It was first put forward

by Kingsbury¹³⁶ in 1912. This hypothesis was based principally upon the fact that fat solvents such as acetone, acetic acids, and alcohol, which reduce the respiration rate of cells, also dissolve the mitochondria. They also dissolve the Golgi apparatus and probably affect other cell membranes, so there seems no reason why the mitochondria any more than the Golgi apparatus should be regarded as the respiratory centres. A more recent protagonist of the respiratory hypothesis of mitochondrial activity is Joyet-Lavergne^{130,131}. This author finds that mitochondria have considerable oxidizing and reducing power. This he believes to be due to a 'redox' system composed of vitamin A and glutathione. For example, he says that mitochondria oxidize leuco-derivatives of certain dyes and that they can oxidize cobaltous salts to cobaltic salts. He states that he has used the following substances to demonstrate the reducing power of mitochondria: gold chloride, silver nitrate, chromic acid, picric acid, potassium permanganate, *m*-dinitrobenzol. He quotes Chodat and Rouge⁵⁰ as having shown that oxidase and peroxidase reactions are given in the region of mitochondria, but he points out that Prenant¹⁸⁵, Hollande^{113,114}, and Mangenot¹⁵⁸ showed that this was so only in certain cells and that the presence of oxidases and peroxidases was not a property of all mitochondria. Although Joyet-Lavergne^{130,131} believes that the respiratory property of mitochondria is dependent upon a 'redox' system made up of vitamin A and glutathione, there is no evidence that such a system plays a part in any of the known respiratory systems of cells. As direct evidence that mitochondria play a part in respiration he states that young red blood cells of vertebrates have a higher respiration rate, and have more mitochondria, than old red blood cells. So many changes, however, must take place in a red blood cell when it ages that one can hardly claim *per se* that the change in respiration rate is due to the change in the number of mitochondria.

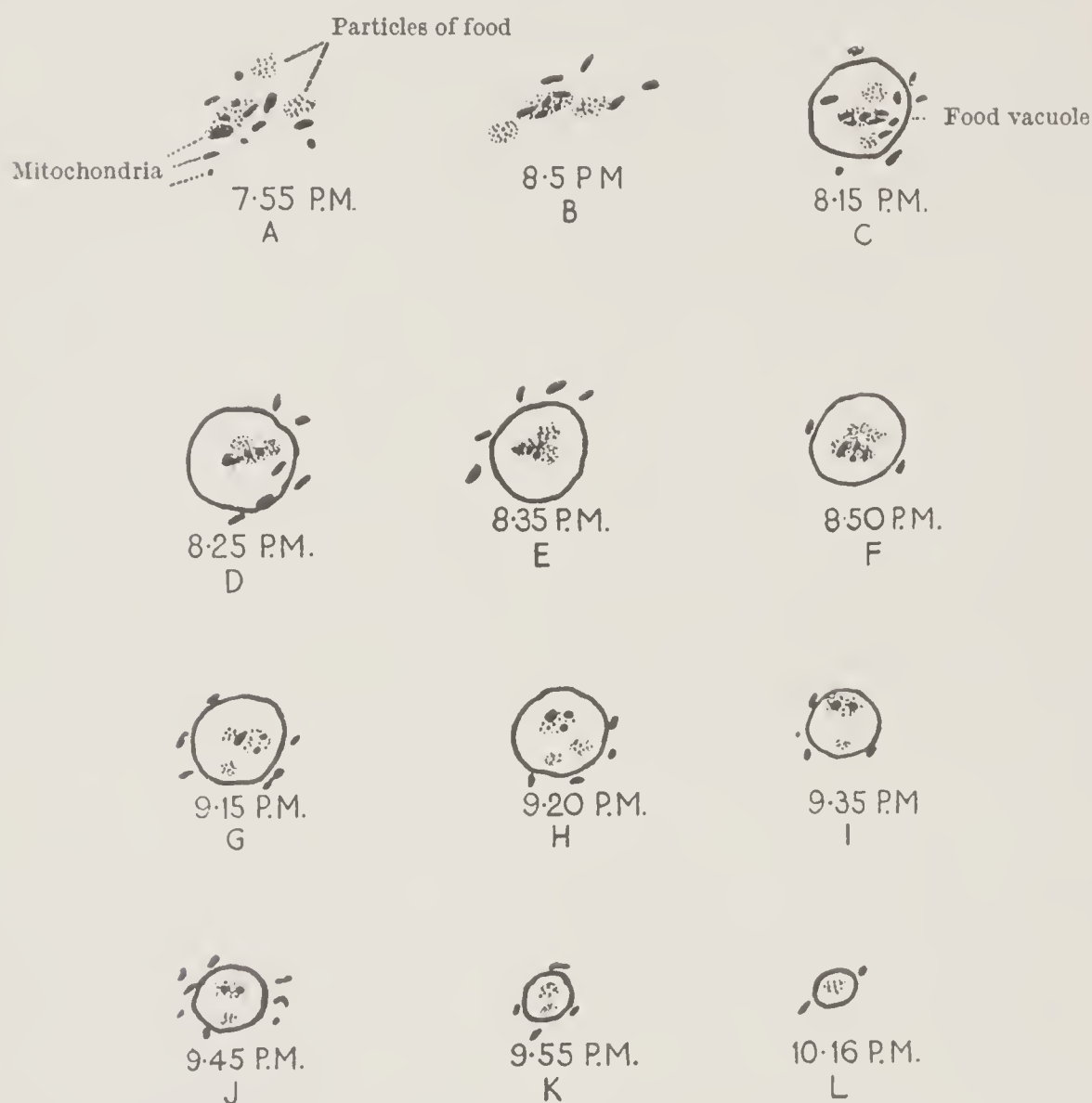
On the other hand, the recent discoveries by Hogeboom^{111,112}, Claude^{53,54}, &c., of the presence of the major part of the succinic dehydrogenase and cytochrome oxidase activity in the mitochondria seems to support the view of the early workers on these organelles—it does appear that they may be the chief centres of aerobic respiration of the cell.

Considerable evidence has been brought forward by Horning that mitochondria possess proteolytic enzyme activity, or rather that such activity occurs at the mitochondrial/cytoplasmic interfaces.

In protozoa, Horning^{117,118,119} has demonstrated both the lytic and synthetic activity of mitochondria.

In amoeba¹¹⁷ he found that pieces of engulfed food circulate in the cytoplasm, and that in this process they come into contact with mitochondria, which stick to them. A vacuole is then secreted around the food particle and its associated mitochondria. Other mitochondria were found to aggregate on the outside of the vacuole. As digestion proceeds

the food particles break up, and at the same time the mitochondria become smaller and smaller and gradually pass into solution (Text-fig. 4). Horning and Petrie¹²² have also found some evidence from the germination of cereals that diastase appears to be secreted in association with



TEXT-FIG. 4. Digestion of food particles by mitochondria in amoeba. Re-drawn (modified) from Horning¹¹⁷.

the mitochondria. The mitochondria appear to be secreted by the scutellar cells into the starch-containing cells of the endosperm; they become applied to the starch grains and result in active hydrolysis. As the starch grains erode the mitochondria disappear.

In the symbiotic protozoön, *Opalina*, Horning¹²⁰ finds a concentration of 'vegetative' material at the surfaces of the mitochondria. At a certain stage the granules break from the mitochondria and lie free in the cytoplasm (Plate 1, Fig. 3). Horning believes that these granules represent protein material which has been synthesized under the influence of the mitochondria.

Horning is of the opinion that mitochondria are responsible for the production of the zymogen granules of the pancreas and that here,

instead of producing enzymes for intracellular digestion they produce enzymes to be used for extracellular digestion, and thus their activity in the lower and higher forms of animal life is fundamentally the same.

Levi and Chevrement¹⁴³ believe that myofibrils have a mitochondrial origin, and Foster⁸² has summarized their relationship to endocrine function.

There is some evidence in the literature that mitochondria might be concerned with fat metabolism.

Such a suggestion was made by Wotton²³⁷ for the mitochondria of trypanosomes. A similar claim was made by Wimsatt²²⁹ who found that in bats there was an inverse relationship between the occurrence of fat and mitochondria in the cells of the glandular epithelium and stroma of the uterus and in those of the yolk sac endoderm. A similar relation has been found by other authors. The finding by Hartman¹⁰³ that there is an increase in the number of mitochondria in the ventral horn cells of the rat following section of the sciatic nerve suggests that they may be concerned with the regeneration of myelin, a fatty lipid complex.

The most important evidence for the relationship of mitochondria to fat metabolism comes firstly from Bensley's work (1947)²³ on the pigments of the mitochondria and the submicroscopic granules of the liver, and secondly from the work of Hogeboom *et al.*^{111, 112} on the presence of fatty acid oxidase in mitochondria. Bensley isolated from mitochondria a deep-red pigment which in solution in acetone absorbed the whole of the ultraviolet and much of the blue end of the spectrum. This pigment was believed to be identical with that produced by the slow oxidation of lecithin. Weiss²²⁶ had previously found a similar pigment to be produced by the oxidation of fats obtained from yeast. Bensley believes that the pigment found in liver mitochondria results from the oxidation of unsaturated fats, and particularly of phospho-lipines, and he concludes that in the liver cell the mitochondria and the submicroscopical particles are the seat of highly active oxidative processes involving the metabolism of fats. Bensley suggests that mitochondria, instead of being regarded as semi-permanent self-reproducing cell organelles, should be thought of as simply temporary aggregates of complex composition, containing vitamins, enzymes, and so on. He points out that although lecithin is actually synthesized by the liver cell it always seems to be bound to the structural components of the cell. He believes, therefore, that as lecithin is formed it is rapidly incorporated into existing mitochondria or granules or is used to form new, but similar, elements. In confirmation of this conception he quotes the fact that in prolonged inanition mitochondria rapidly disappear from pancreatic cells, but return again when feeding is resumed.

It may well be, as Bensley finally suggests, that mitochondria really represent a reserve store of metabolic material for the cell. The presence

of vitamins, enzymes, &c., in these structures may simply be due to the fact that they tend to aggregate at surfaces, and that mitochondria happen to provide a suitable but temporary surface on which this may occur.

d. Relation of Mitochondria to the Golgi Apparatus

A detailed discussion of this problem is given by Hirsch; it is therefore not necessary to deal fully with it here. It is of interest, however, to quote briefly the views of Hirsch and Ludford on this relationship.

According to Hirsch¹⁰⁹ there are two groups of views on this question. One group asserts that there is a direct transition of mitochondria into Golgi material, and the other group postulates the liberation by mitochondria into the cytoplasm of various substances which pass to the Golgi apparatus. Ma¹⁵³, however, believes that the Golgi apparatus is changed in gland cells into secretion granules and mitochondria. Most cytologists, however, do not agree with this view. Other workers have claimed that mitochondria form either the internal or the external part of the Golgi apparatus. Hirsch in general does not favour the view that the mitochondria change into the Golgi apparatus, but the fact that his Golgi 'pre-substance' stains with Janus green B and neutral red suggests that it may be intermediate between the two organelles.

That there is a connexion between mitochondria and the Golgi apparatus is shown rather strikingly by Hirsch on the living pancreas. He found that small granules were formed on the surface of the mitochondria. These granules remained in contact with the mitochondria for a time varying between 10 and 70 minutes, and then they became detached and moved towards the Golgi field at a speed of $1\ \mu$ every 6 to 8 minutes (Plate 2, Fig. 8). Duthie^{74,76} has seen a somewhat similar picture in fixed preparations of salivary glands and goblet cells of the intestine. Ries¹⁹⁰ has confirmed the work on the living pancreas. There is evidence that in other cells mitochondria give off material which is absorbed by the Golgi apparatus. Ludford¹⁴⁸ has stated: 'At the mitochondrial/cytoplasmic surface syntheses by enzymes occur. The resulting products continually diffuse into the cytoplasm preventing an accumulation at the surface of the mitochondria which would inhibit further synthesis. At the surface of the Golgi apparatus the elaborated products are concentrated into droplets preliminary to their elimination.' Hirsch¹⁰⁹ sums up the problem in a similar fashion with the following statement:

Dennoch stehen beide Systeme in einem nahen physiologischen Zusammenhang; es sei, daß Produkte von Mitochondrien als solche direkt in Golgi-Körper übergehen; es sei, daß Stoffe oder Fermente, welche von den Mitochondrien gebildet worden sind, weitergegeben werden an die Golgi-Systeme um hier eine endgültige Verarbeitung zu erfahren oder bei dem Aufbau von Stoffen in den Golgi-Systemen als Katalysatoren zu wirken.

Wie der physiologische Zusammenhang zwischen Mitochondrien und Golgi-Systemen im einzelnen Falle sich abspielt, muß noch exakter experimentell untersucht werden.

e. Summary of Mitochondria

Mitochondria are cell organelles of variable length but of constant diameter in individual cells. Liver mitochondria are composed basically of protein, fats and fatty acids, phospho-lipins and sterols. They may contain vitamin A and/or carotene, vitamin C, members of the vitamin B complex, glutathione, proteolytic and respiratory enzymes, and oxidases.

The composition of mitochondria appears to vary in different tissues under different conditions, and is affected by pathological changes in the cells.

It is likely that mitochondria can be divided into an outer and an inner portion, the fatty substances being concentrated in the former, and protein being present in both. It is probable that they are surrounded by an adsorbed layer of protein which prevents them from being stained by fat dyes.

It may be that mitochondria represent reserve stores of metabolic material for the cell, particularly in view of the fact that they are reduced in inanition.

It is possible, in view of the localization of respiratory enzymes in mitochondria, that Kingsbury's view, first expressed in 1912, that they are respiratory centres of the cell may approach the truth.

Mitochondria may be concerned with fat metabolism and with proteolytic and, in the case of germinating seeds, with diastatic activity.

iii. THE GOLGI APPARATUS

a. General

It is impossible in the space available to analyse the vast amount of controversial literature on the Golgi apparatus. It is even impracticable to mention all the various aspects of the subject. Comprehensive reviews are, however, in existence and have been mentioned in the introduction to this chapter. It is hoped to discuss fairly fully some more recent aspects of the study of the Golgi apparatus, and to offer a few tentative conclusions as to the function of this 'most protean of all cytoplasmic inclusions'¹³⁸.

Cowdry, writing in 1924⁶¹, said of the Golgi apparatus:

Even now, 25 years after its discovery, we can only say that the Golgi apparatus is an area of the cytoplasm frequently (especially in higher forms) of reticular shape, often as large as the nucleus, and sometimes definitely located with regard to cellular polarity. Part of the material of which it is composed is soluble in alcohol, becomes blackened after prolonged treatment

with osmic acid, and, after appropriate preliminary fixation, shows marked affinity for silver salts. In addition it may occasionally be stained with resorcin fuchsin, iron haematoxylin, and other dyes, but the word 'apparatus' is unfortunate because it carries with it the idea of a mechanism of rather mechanical type.

It is now more than fifty years since Golgi⁹⁶ discovered the internal reticular net in the nerve cells of the barn owl and cat. These nets had actually been observed before Golgi (1898) by Platner, 1885¹⁷⁹, and Hermann, 1891¹⁰⁶, and for the first twenty-five years after their discovery investigation of the Golgi apparatus was almost entirely of a morphological nature. During the last ten or fifteen years, however, a great deal of attention has been paid to its function and composition.

During the early stages of the investigation of the Golgi apparatus the subject was confused by the claim of Holmgren¹¹⁵ that it was identical with a system of clear canals which he claimed to have discovered in many cells and which he called the 'trophospongium'. Cajal^{45,46} added to the confusion by referring to the Golgi nets as Golgi-Holmgren canals. The general opinion of cytologists now is that there is no connexion between the two systems, and the recent evidence obtained from ultracentrifugation of cells, which shows that the Holmgren canals become stratified in a different position from the Golgi apparatus, seems to have dealt the theory a death-blow.

Later, the 'vacuome theory' of the Golgi apparatus was suggested. Originally propounded by Accoyer¹, it was elaborated by Parat and Painlevé¹⁷⁵. The theory was described by MacBride and Hewer¹⁵⁴ as follows:

All animal and plant cells have two fundamental but independent morphological elements—the vacuome and the chondriome. The vacuome is an aqueous phase; the chondriome a lipoidal one. The vacuome consists either of isolated vacuoles or else of canalicular systems. The vacuome stains specifically with neutral red *intra vitam*. From these premisses they state that the 'reticular apparatus' of Golgi and the 'trophospongium' of Holmgren (in fact the whole classical Golgi apparatus) are artefacts produced by precipitation of silver or osmium at the surface of, inside, or between, the vacuoles.

Parat later modified his theory to include dictyosomes, and announced that the dictyosomes were scale-like cortices of the 'vacuome' and introduced for them new names, 'lepidochondriosomes' and 'lepidosomes'. He further stated that the 'lepidosomes' were merely modified mitochondria, and introduced still another and third name for them, 'chondriome actif'. The cell thus contained a 'chondriome' and a 'chondriome actif' (or lepidosome) which was associated with the vacuome. The vacuoles in the animal are homologized with the large intracellular vacuoles of plants principally because, according to the protagonists of the theory, they both stain with neutral red.

Parat's views did not find favour with the majority of cytologists. Gatenby⁸⁶, for example, commented that not all neutral red bodies were argentophile, whereas the true Golgi apparatus always was. Some doubt was also cast on Parat's view by the fact that in some cells at least neutral red appears to produce new vacuoles in the cytoplasm which, according to Hirsch¹⁰⁹, represent the crinome of Chlopin⁴⁹. Hirsch claims that neutral red stains his 'pre-substance' but not the Golgi system. The whole subject of 'Neutral Red' cytology has been vigorously attacked by Gatenby⁸⁸, and his essay should be read by all serious students of the cell as summarizing in a succinct fashion the case on behalf of Parat's critics.

However, there is evidence that neutral red vacuoles do occur in association with the Golgi apparatus. For example, Covell and Scott⁵⁷ observed slow blackening of neutral red granules aggregated in the Golgi region of nerve cells of a ventral horn smear as a result of the introduction of osmium tetroxide under the cover-glass.

The Oxford school of cytology has been tackling the problem of the morphology of the Golgi apparatus and the relation to it of the neutral red vacuoles. Their results are considered in detail at the end of the following section.

The Golgi apparatus, or some representative of it, can be found in every vertebrate and invertebrate cell. The work of Bowen³⁶ indicated that the Golgi apparatus existed in plants in the form of osmiophilic platelets. There are no records of its occurrence in algae, fungi, or bacteria. Protozoa are known to possess a Golgi apparatus. It has been specially investigated by Horning^{117,118,119,120}, Horning and Richardson¹²³, and by Kedrowsky^{132,133}, in the case of *Opalina*, the intestinal protozoan symbiote of frogs.

Smyth^{202,203} found osmiophilic material in some ciliates associated with the contractile vacuole. He has suggested that the osmiophilic material round the contractile vacuole is Golgi material and that it is homologous with the metazoan Golgi apparatus.

Sosa²⁰⁶ has suggested a nomenclature (see below) for the Golgi material which would do something (if adopted generally) to reduce the present complexity of names used for this organelle.

Golgiokinesis. Division of the Golgi apparatus during karyokinesis.

Golgiosomes. Corpuscles produced by Golgiokinesis. Structures present in invertebrates and described as Golgi material.

Golgiolysis. Process of dissolution of the Golgi apparatus.

Golgiorrhesis. Fragmentation of the Golgi apparatus.

Golgiogenesis. Formation and differentiation (ontogenetic) of the Golgi apparatus.

Golgio-cytoarchitecture. Study of the cytoarchitecture in relation to the Golgi apparatus.

b. Structure and Physical Condition of the Golgi Apparatus

Kirkman and Severinghaus¹³⁸ state that the Golgi apparatus has been described at various times as consisting of 'a fibrous reticulum, network, ring, or cylinder, a very irregular fenestrated plate, a more or less incomplete hollow sphere, vesicle, or cup, a collection of small spheres, rodlets and platelets or disks, a series of anastomosing canals, a group of vacuoles, and a differentiated region of homogeneous cytoplasm crossed by irregular interfaces'. The apparatus very frequently occurs as a network, and many authors have questioned the validity of such a structure. Hirsch¹⁰⁹ denies the existence of most networks, and other workers have regarded them as staining artefacts. It is of interest that grains of reduced silver seem to be composed fundamentally of a network structure. The granules of silver in a photographic emulsion even under the high power of the ordinary microscope appear to be homogeneous, but when they are examined with an electron microscope, magnifying them by 25,000 diameters, they may be seen to be networks, made up of threads, some of which are only five atoms thick (Plate 2, Fig. 2). The type of network can be varied according to the developer used. Hydroquinone produces coarse threads, whereas metol produces fine threads. With some other developers the network becomes a sort of fluffy mass. The form of the Golgi network also varies according to the time of development and the type of developer used.

In various cells the Golgi apparatus varies considerably in size and shape, and is usually well developed in the stage of cytomorphosis, and tends to decrease in size as the cell becomes older. In gland cells the apparatus is noticeably large, but it is relatively quite small in muscle cells. Actually the problem of making accurate comparative estimations of the amount of Golgi apparatus is most difficult, because, as with mitochondria, even with the most accurate standardization of technique, unaccountable variations may occur. Nevertheless it has proved possible to measure the Golgi apparatus in relation to cell size in the uterine glands of rabbits.

Pollister¹⁸² reconstructed the Golgi apparatus from serial sections of single cells and showed that it appeared as a platework of irregular shape and uniform thickness.

As a general rule in vertebrate somatic cells the Golgi apparatus is in the form of a dense network situated near the nucleus; it may, however, vary according to the physiological state of the cell. The general structure varies in cells of different organs, although the shape is never identical even in the neighbouring cells of the same organ. In developing germ cells the apparatus may be in the form of rods or granules. This is also the condition in which it is found in most invertebrate cells. Horning¹¹⁹, however, has found Golgi nets in the cells of *Hydra viridis*.

In mitosis the Golgi apparatus usually breaks up into small particles or granules, which are distributed more or less evenly through the cytoplasm. Cytoplasmic division causes an approximate halving of the Golgi substance. Ludford¹⁴⁶ has described five different types of division of the apparatus.

The behaviour of the apparatus in gland cells emphasizes that we are dealing with a cellular component whose existence cannot be denied. Kirkman and Severinghaus¹³⁸ state:

The most conclusive proof that the Golgi apparatus is an organelle is not the universality of its presence in properly fixed material, nor even the descriptions of it in living material, but rather its behaviour as determined by microscopical observations of it in the same type of cell in different physiological conditions. This behaviour is so characteristic and so constant that there should be no question concerning the reality of the substance or structure exhibiting it.

That the Golgi apparatus consists of two parts—an outer, which absorbs osmium and silver, and an osmic- or argento-phobic inner portion—has been claimed by a number of workers (including Bowen¹³²; Weier²²⁴; Richardson¹⁸⁹; Uhlenhuth²¹⁷; Bourne²⁷; Hirsch¹⁰⁹). This double structure is beautifully shown in Plate 2, Fig. 3 (taken from Richardson¹⁸⁹). Hirsch has found that the Golgi bodies of the cell do not always have this double structure, and he has recently devoted a book to a consideration of his pre-substance-Golgi-system theory. Hirsch declares that the solid granules of Golgi substance, which show no differentiation into external and internal portions, really constitute the 'pre-substance' of the Golgi apparatus. These pre-substances are able to build up nets, but they are the only sort of nets that Hirsch recognizes. The other nets, he claims, are due to over-impregnation of separate bodies by excessive amounts of osmium or silver, which causes them to link up and simulate a net. These pre-substances may be aggregated near the nucleus or distributed throughout the cell. Each piece of pre-substance gradually develops a double structure, with an argentophile and osmiophile cortex; at this stage it is referred to as a Golgi system. A number of joined Golgi systems are known as 'polysystems'. The outer part or cortex of the Golgi system is known as the 'externum', the inner part as the 'internum'. Hirsch¹⁰⁹ believes that the product* of the cell is formed in the internum; he also gives the impression that only the pre-substance stains with neutral red and only the Golgi system with trypan blue. He claims, further, that the pre-substance stains with Janus green B.

Some information as to the physical state of the Golgi complex is given by ultra-centrifuge studies (Beams, King, Dornfield, and others). They show that in most cells the specific gravity of the Golgi material

* The 'product' of the cell is the term used by Hirsch to describe the granules of secretion which are so obvious microscopically in externally secreting glands.

is less than that of most of the other cellular constituents. In uterine gland cells the Golgi complex is of a very fluid consistency. In thyroid cells (Hellbaum¹⁰⁵) and in spinal ganglion cells (Brown⁴⁰) the Golgi complex retains its form even though displaced from its normal position in the cell; it seems, therefore, to be relatively rigid in the cells of these organs. Dornfield^{72,73} has shown that in transplants of centrifuged adrenal cells the displaced Golgi material returns to its normal position in the cell after a short time and becomes indistinguishable from that of normal adrenal cells.

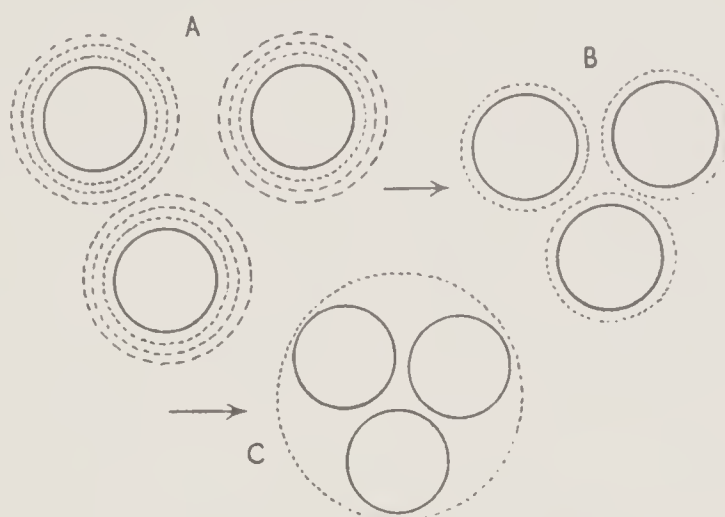
Pollister¹⁸¹ suggests that the Golgi apparatus could not be fluid or even a highly plastic solid and still maintain its plate-like form, although he found it elastic enough to bend under pressure and to straighten out again when the pressure was removed. Simpson, on the other hand, believes it to be in a highly fluid condition.

The Golgi complex probably varies in consistency in different cells of the body and probably varies in individual cells according to their physiological condition. The Golgi apparatus in developing male germ and other cells is frequently associated with a differentiated area of cytoplasm near the nucleus, called the idiozome, which may stain differently from the rest of the cells.

The interior or 'internum' of the Golgi apparatus seems to be a water-rich area similar to the internal region of the mitochondrion, into which water-soluble substances (e.g. vitamin C) may be passed. The evidence available suggests that the homogeneous bodies (pre-substance of Hirsch) and the osmiophilic portion of the Golgi system are composed of lipoprotein. These homogeneous bodies may, as Hirsch has suggested, be converted into the Golgi systems by the absorption and concentration into the interior of water and water-soluble substances and by the activity of enzymes which produce the product of the cell within the interior of the homogeneous body. It is possible that the pre-substance may contain a small central core, but that it cannot be seen because it is obscured by a thick, heavily impregnated outer wall. It seems possible that since the 'pre-substance' stains with Janus green B and neutral red it contains proteolytic enzymes. The fact that the Golgi apparatus can hypertrophy, and the possibility that it contains enzymes, suggest that it is able to convert cytoplasm of the cell into extra enzyme material in the same way that some virus particles can convert the tissues of the host into fresh virus material. This implies that the Golgi apparatus can convert the elements of the cytoplasm into material which possesses more free energy than they possessed before. This sort of change, however, is not unusual when we are dealing with living tissues, because it is carried out by every organism when it converts food into living material.

Hirsch¹⁰⁹ has listed points of resemblance between the Golgi apparatus

and coacervates. The term 'coacervate' comes from the Latin *acervus*, a crowd or swarm, and it refers to the structures formed during the slow separation of a colloid from solution. Coacervates are formed of a large number of small droplets which lump together. In a typical colloidal solution each particle is surrounded by concentric sheaths of water. The inner layer is very firmly bound to the particle, and the successive



TEXT-FIG. 5. Various stages in the formation of a coacervate. The dotted lines represent watery shells around the colloidal particle.

water layers are less and less strongly bound until there is a gradual gradation into the free water of the sol. One can conceive each colloid particle, therefore, as being surrounded by an envelope of water. Change of electric charge on the colloidal particles, or the addition of dehydrating agents, causes the removal of the envelope of loosely bound water, leaving only the strongly bound water attached to the particle. In this condition the particles come into contact with one another, and the various water shells join up (Text-fig. 5).

Hirsch makes the following comparisons between coacervates and Golgi systems.

1. Coacervates are stable systems which are in equilibrium and do no work. On the other hand, Golgi systems do perform work.
2. We know the chemical composition of the various coacervates which have been formed, but we do not know for certain whether the chemical substances of the Golgi system are those which could form coacervates.
3. The form of coacervates and Golgi systems is similar.
4. The state of the Golgi system changes, varying from liquid to solid. It is possible by varying the external conditions to produce similar changes in the consistency of the coacervates.
5. Various substances can be adsorbed on the exterior of the Golgi systems. Coacervates also possess this ability.

6. An externum and internum are present in both Golgi systems and coacervates, but the externum is often very irregular in the case of the Golgi system.

We see in this comparison some striking similarities between Golgi systems and coacervates, but probably the greatest argument against such homology is the first quoted comparison, i.e. that the coacervate is a relatively stable system and the Golgi apparatus has free energy and is able to do work. If the Golgi system is a coacervate it is one which is very different from any of those with which we are familiar.

The Golgi material of the spermatocytes of molluscs (Monné¹⁶⁹), when examined under polarized light, shows a positive birefringence in the peripheral (chromophilic) portion. This suggests that in these regions the molecules are arranged radially. This birefringence is increased by staining with dyes such as rhodamine B (see also Hibbard and Lavin¹⁰⁸). Golgi material in tissue-culture cells treated with osmium tetroxide is shown by the electron microscope to have a particularly dense structure. This high density combined with osmic impregnation makes it impossible to see details of the internal structure of the material (Porter *et al.*¹⁸⁴). Sosa²⁰⁶, however, has shown that the Golgi material of mononuclear leucocytes appears to be, by the electron microscope, less dense than the remainder of the cytoplasm, and appears to have a reticulated structure.

Hibbard¹⁰⁷ has pointed out that the typical network of fixed preparations has never been seen in a living cell, and quotes a personal communication from W. H. Lewis that he has seen no sign of the classical Golgi apparatus in any living tissue-culture cell. For the same reason Strangeways and Canti²⁰⁷ denied that the Golgi apparatus existed. Chambers, in his micro-dissection studies, was unable to find anything which suggested the presence of a membrane or vacuoles in the Golgi region of the cell.

According to Ludford¹⁴⁷ the reason why no Golgi apparatus can be seen in cultured cells is that 'as some cells spread out the Golgi apparatus is stretched until it fragments and its individual particles become dispersed in the cytoplasm'. Richardson¹⁸⁹, however, found a large and compact Golgi apparatus to be present in fixed cultured cells (Plate 2, Fig. 3). Ludford¹⁵⁰ later showed that fragmentation of the apparatus occurred only at certain stages in cultured cells, and that it was because of this that Strangeways and Canti were unable to see any signs of it. Ludford has published photographs of living tissue-culture cells stained *in vitro* with methylene blue in which a distinct Golgi area can be seen near the nucleus. This Golgi area appears to be somewhat reticular in structure (Plate 2, Fig. 1). The nucleus shows as a dark, oval figure near the left side of the white mass. The Golgi area is the dark region speckled with white immediately to the right of the nucleus. Ludford has also obtained photographs of living cells, taken with ultraviolet light, which

show the Golgi region as a half-moon-shaped area adjacent to the nucleus and looking as though it might be reticular in nature (see Chapter 10).

Walker²²⁰ and Walker and Allen²²¹, basing their statements on their experiments with chemical models composed of gelatin, albumen, and lecithin, have endeavoured to show that the Golgi apparatus can be regarded as an artefact. One might concede that their experiments throw some doubt on the existence of Golgi nets, but it is quite unjustifiable to argue from them that the Golgi apparatus is a non-existent structure. Heilbrunn¹⁰⁴ has also been very sceptical about the Golgi apparatus. In 1934 Beams and King^{18,19}, using a centrifuge, designed by Professor J. W. Beams, which was capable of producing a centrifugal force of 400,000 g., showed that in uterine gland cells of guinea-pigs the Golgi apparatus could be removed from its normal position in the cell. Gresson⁹⁷ has also found stratification of the Golgi bodies of mouse oocytes after centrifuging. These results confirmed that the Golgi apparatus was a definite cell organ and, since it was thrown centripetally, that it was less dense than the cytoplasm. Since Beams and King found that there was no displaced system of canals or vacuoles, their experiments dealt a severe blow to the vacuome hypothesis.

Hirsch¹⁰⁹ has pointed out that the inability to see the apparatus in most living cells is no indication that it is not present, because, he says, it has an identical refractive index with that of cytoplasm. Hirsch claims that it is impossible to see the nuclei in the living cells of the pancreas of the white mouse even under the best conditions (this is denied by Ludford: personal communication), but that nobody would say that the nucleus which is apparent after fixation and staining is an artefact. He also states that the assertion by many workers that the Golgi apparatus is never visible in living cells is wrong. For, apart from Ludford's observations, van Bergen²⁶ saw the apparatus in the unstained prostate gland. Hirsch and Bretschneider¹⁰⁹ have seen the Golgi apparatus in the living intestinal cells of *Ascaris*. Gatenby has seen a Golgi apparatus in the unstained living cells of invertebrates, and Macdougald and Gatenby¹⁵⁶ claim to have seen the apparatus in living Purkinje cells of mammals and in spermatocytes. Certain ectosomal granules of *Opalina* are also visible in life, and these are regarded by some as forming part of the Golgi apparatus. The apparatus has been seen in living eggs, and in some cells it can be brought into view (Hirsch¹⁰⁹) by the use of neutral red or trypan blue or other dyes, or by varying the acidity of the medium in which the cell is immersed.

One might have expected that the introduction and general use of phase-contrast microscopy (see Chapter I, Section C) would have led to the identification of Golgi nets (if they exist) in numbers of living cells, but even this all-revealing form of microscopy has failed to demonstrate

net-like structures in the cell. Brice, who has examined the living spermatocytes of *Lumbricus* by phase-contrast microscopy, found the Golgi material to be composed of a typical sphere with a light centre comparable with osmiophilic and osmiophobic regions. In some cells the apparatus was in the form of a dark crescent resting on a clear sphere and closely applied to the nucleus.

In a variety of living cells Barer and Crawford, working at Oxford with phase-contrast, have been unable to detect anything resembling a net in the Golgi region (personal communication), and Baker¹¹ and Thomas^{210,211}, also at Oxford, in a careful study of the Golgi regions of various cells, and using the same form of microscopy, were likewise unable to discover networks.

One is now in the position of being more or less forced to accept the fact that the reason why earlier workers failed to see the Golgi apparatus in living cells was that they were looking for the typical network structure of the fixed impregnated cell, and that it is possible that such structures do not occur in life.

Worley²³⁵ and Worley and Worley²³⁶ found that the Golgi apparatus in mollusc cells has a typical vesicular form, and they pointed out that these vesicles may be ruptured in handling and various treatment, and that the contents escape and adhere to the surface of nearby bodies, e.g. yolk, or they may form batonettes, and may even flow on to unruptured vesicles. As a result the apparatus may change from its appearance as granules, droplets, vesicles, or masses in the living cells to rings, plates, cups, disks, &c., in the dead cell.

In another paper (1944) Worley²³⁴ stated that in vertebrate cells the Golgi apparatus had a simple vesicular form approximating that of mollusc cells. He found the apparatus to consist of a number of chromophilic vesicles within each of which was a colourless or chromophobic region. This he believed was the developing product. The vesicles, he found, under conditions of activity, sometimes elongated and came into contact with each other.

In 1944 Baker¹¹ made a significant contribution to our knowledge of the Golgi apparatus with his 'Structure and Chemical Composition of the Golgi Element'. Baker examined the apparatus after various staining methods in the primary spermatocytes and early spermatids of the common snail (*Helix aspersa*), the absorptive cells of the intestine of the newt (*Triturus vulgaris*), and the nerve cells of the anterior mesenteric ganglion of the rabbit.

He found the Golgi apparatus was, in general, composed of four parts:

1. Neutral red vacuoles.
2. Dense lipoid-containing substance.
3. Diffuse lipoid-containing substance.
4. Golgi product.

The vacuoles he found to be spherical and to contain not enough protein for them to have a granular appearance after fixation—in fact, they were usually destroyed by fixative mixtures. These vacuoles have a considerable segregating power for certain dyes; that is, they can concentrate the dyes from dilute solutions. The dense lipoid substance could invest the vacuoles completely, appear as irregular strands spreading between and over the surfaces of vacuoles, or could be attached to individual vacuoles as caps or crescents or rods. The vacuoles and the dense lipoid were found to be immersed in a diffuse lipoid substance. The Golgi product was found to arise within the vacuoles.

In cells which were not actively functioning he found the vacuoles to be either not present at all or to be so small that they could not be demonstrated. The Golgi apparatus then became simply a mass of lipoid which was equivalent to or identical with Hirsch's pre-substance. Activity of the Golgi apparatus resulted in the appearance of neutral red vacuoles.

The presence of neutral red vacuoles in the region of the Golgi apparatus has also been shown by Hibbard¹⁰⁷ in the cells of the digestive tracts of young and embryonic chicks.

Thomas^{210,211}, working in Baker's laboratory, has investigated with great care and detail the Golgi apparatus in the neurones of *Helix aspersa* and the sympathetic neurones of the superior mesenteric ganglion of the mouse. He used both phase-contrast and the more usual microscopical techniques. Here again he found that the living cells contained spheroid bodies with a chromophobic core and a lipoidal pellicle. These bodies he describes as discrete binary systems. The cores of these spheroids, which stained with neutral red, are, he points out, comparable with Parat's 'vacuome'. The most assiduous study of the cells with phase-contrast showed no signs of a network or of the classical dictyosomes which are demonstrated by the standard impregnation techniques.

Thomas found that these spheroids are tinged brown by osmic vapour and that if the cell is immersed in osmic acid (osmium tetroxide) they become blackened. Prolonged osmification blackens mitochondria and these link up with the spheroids to form nets.

Gatenby, however, claims that there is a separate Golgi apparatus apart from the structures described by Thomas.

Baker's work suggests, nevertheless, that in certain cells it is highly unlikely that the Golgi apparatus is in the form of a conventional net. It may be that all Golgi nets are impregnation artefacts, although the recognition of the presence of diffuse lipoid has made it possible that metallic impregnation would produce a net-like appearance which is due entirely to the Golgi material, and not to the over-impregnation of other cytological constituents.

One fact which is now unequivocal is that Golgi nets identical with

those shown in fixed preparations cannot be seen in most living cells even by the most modern microscopical techniques. If such nets exist in life they may be made up of some substance which diffuses at its edges into the surrounding cytoplasm and has no well-defined boundary or membrane.

The fact that Baker¹¹ and Foster⁸³ have shown that Golgi vesicles in different cells are embedded in diffuse lipoid would explain both the demonstration of nets by metallic impregnation (due to deposition of the metal in the diffuse lipoid between vesicles) and the apparent absence of such nets in living cells.

c. Composition of the Golgi Apparatus

Nath (quoted by Hirsch¹⁰⁹) has stated that the Golgi apparatus can be demonstrated in eggs by treating them with 2 per cent. osmium tetroxide for 10–15 minutes. This capacity on the part of the apparatus to reduce osmium tetroxide even without prior fixation has led many authors to claim that the Golgi apparatus contains unsaturated fats and fatty acids. This is not necessarily so, for it appears that the Golgi apparatus may contain vitamin C, which reduces osmium tetroxide; and Lison¹⁴⁵ has pointed out that the latter demonstrates a number of reducing substances—a point which has also been made by Owens and Bensley¹⁷⁴. Most techniques, however, which are used for demonstrating the Golgi apparatus with osmium tetroxide are used after fixation with reagents which would destroy vitamin C and therefore in these cases the reduction of osmium tetroxide may indicate the presence of unsaturated fatty material. In some cases fat dyes have been used to stain the Golgi apparatus (Cowdry⁵⁸; Weiner²²⁵), but this is rare. The fact that the apparatus is soluble in the usual fat solvents suggests that it may contain a high percentage of fatty or lipoidal material, and Watzka²²³ has claimed that the Golgi material breaks up after chloroform narcosis. Monné has shown that if the germ cells of *Helix* are treated with sodium bicarbonate myelin figures are formed from the dictyosomes. On the other hand, Thomas^{210,211} says that the dictyosomes in *Helix* are not Golgi material and that they are formed by the over-impregnation of mitochondria. Ciaccio was able to stain the Golgi apparatus in spermatids (mammalian) with his lipoid method, and Boyle³⁷ said that the chromophobe element of the Golgi apparatus of *Helix* neurones was stained by Sudan IV.

Baker¹¹, in a critical discussion of the chemistry of the Golgi 'element', points out that the claim that this body contains fat or lipoid is not based on any valid histochemical evidence. He presents, however, the results of a number of histochemical tests as applied to spermatocytes and spermatids of the common snail, the absorptive cells of the intestinal epithelium of the common newt, and the nerve cells of the anterior

mesenteric ganglion of the rabbit. He found that the Golgi material in these cells contained lecithin, cephalin, or sphingomyelin, and that Windaus's test for cholesterol and Schultze's test for cholesterol and esters were negative. He found the Golgi material to be uncoloured in living tissues, suggesting, therefore, that it contained no carotene. Cain⁴³, however, found carotenoids to be present in the Golgi apparatus in *Limnoea* and *Planorbis*. Cain likewise has been able to confirm that Baker's test for lipines was positive in the region of the Golgi apparatus of the intestinal cells of the leech.

There thus appears sufficient histochemical evidence for the conclusion that the Golgi complex does, in fact, contain lipoid material. The term 'lipoid' as defined by Cain includes 'fats and all other substances that occur in animals and plants and resemble fats in solubility'. Lipine he defines as 'lipoids that yield fatty acids, phosphoric acid, or galactose, and a basic N compound'. Cain confirms that the Golgi material contains *lipine*.

In 1925 Nath¹⁷² suggested that the Golgi material contains protein. He based this suggestion on the fact that it is not completely destroyed by fixing the tissue in Bouin's fluid. Bowen also has suggested that protein is present in the apparatus, and Gatenby is of the opinion that the Golgi material is a combination of protein and lipoid. Ciaccio^{51,52} has advanced a histolipoid theory in which he suggests that some of the lipoid substances contained in animal cells are in loose combination with protein, which prevents them giving the usual lipoid reactions. Ciaccio used unmasking procedures to set free the lipoid. In some cases he used proteolytic enzymes. He was able by these means to demonstrate the idiozomic complex in the male germ cells of *Discoglossus* with fat dyes.

Tarao²⁰⁹ applied this unmasking procedure to the hepatic cells of the mouse and the newt. The livers were first fixed in formalin, then frozen sectioned, and then digested with pepsin and trypsin in turn. They were then stained with aqueous Nile blue sulphate, which is used normally to stain fats and lipoids. Tarao found that the dye had demonstrated the Golgi apparatus. Two photographs (Plate 2, Figs. 4 and 5) taken from his paper show the similarity between the Golgi apparatus demonstrated by this method (Plate 2, Fig. 5) and that demonstrated in the usual way by Kolatchev's osmium tetroxide method (Plate 2, Fig. 4).

Baker¹¹ has pointed out that van Bergen in 1904 stained the apparatus with resorcin fuchsin, that Kirkman had stained it with aniline blue, and that he himself had stained it with nigrosin; and, further, that resorcin fuchsin is also used to stain elastin, and aniline blue and nigrosin are used for staining collagen—both protein substances. These, of course, are not histochemical tests, but Baker gives details of the application of more accurate tests of this nature on the Golgi material of the three types of cells to which he had applied his lipine tests. The Golgi material

was found not to contain arginine or glutathione, but to give a positive reaction with Millon's and the xanthoproteic tests and with the aldehyde test for tryptophane. However, the material was not found to be coloured more intensely than the cytoplasm.

We may therefore conclude that the Golgi complex, like the mitochondria, is composed of protein as well as of lipoidal and fatty substances, but that it contains no more protein than the rest of the cytoplasm.

d. Vitamin C and the Golgi Apparatus

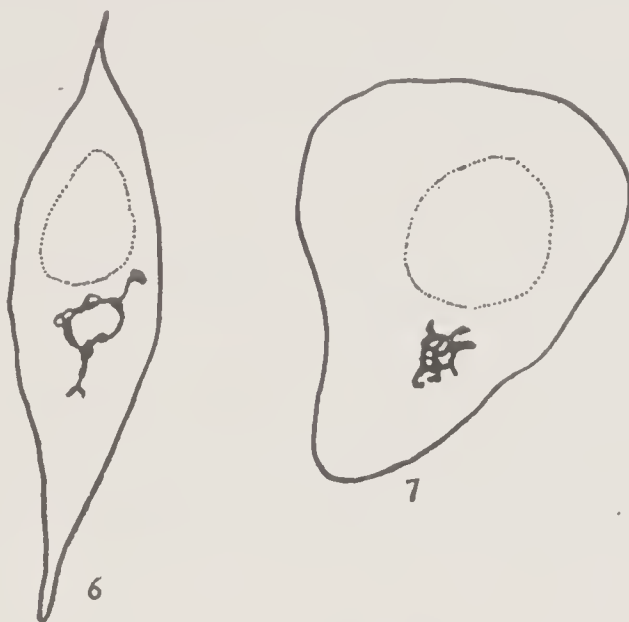
Observations by Bourne^{27,28}, in numerous papers by Giroud and Leblond (see Giroud⁹²), and in a series of papers by Tonutti^{213,214,215}, have suggested that in a large number of cells vitamin C may be associated with the Golgi apparatus. In some cells, e.g. those of the adrenal cortex, vitamin C may occur in the mitochondria instead of the Golgi apparatus, and in other cells, but more rarely (Giroud), it may occur in both mitochondria and Golgi apparatus. Vitamin C is demonstrated histologically by means of a solution of silver nitrate in 10 per cent. acetic acid. (For recent reviews of the specificity of this reagent see Giroud⁹² and Barnett and Bourne¹⁴.)

During the histogenesis of tissues in chick embryos Barnett and Bourne¹⁴ observed that in many cells vitamin C appears to be localized in the region of the Golgi apparatus. This is particularly evident in nerve cells (Text-figs. 6 and 7) where, in addition, it may occur along the developing axon. A particularly striking correspondence is shown in Text-figs. 8 and 9, which demonstrate a vitamin C preparation of the liver of a 10-day chick embryo (Text-fig. 9) and a Golgi preparation (Text-fig. 8; Dalton⁶³) of an 11-day chick embryo. In dividing cartilage cells of chick embryos (Text-fig. 12) the disposition of the vitamin C granules around the chromosomes and the spindle figure is strikingly similar to the disposition of the Golgi apparatus in dividing cells. It is of interest that these cartilage cells were the only dividing cells which showed the presence of vitamin C. Other cells, even if they contained vitamin C before division, appeared to lose it when the time came to divide.

Ries¹⁹¹ has figured the egg of *Aplysia* (Plate 2, Fig. 9), stained to demonstrate granules of vitamin C. It appears that these granules may, according to Hirsch¹⁰⁹, represent Golgi pre-substance, but he is uncertain about it. When the egg starts to divide, the granules pass to certain cells, while others are left without granules. This is a rather different behaviour from what we believe to be characteristic of the Golgi material in dividing embryonic cells. Very little information is available so far on the distribution of vitamin C in the eggs and early embryos of various animals, and this subject would provide a fertile field for research. (For

the distribution of vitamin C in the chick embryo, see Barnett and Bourne^{13,14}).

Miwa¹⁶⁸ has shown that the decrease in size of the Golgi apparatus

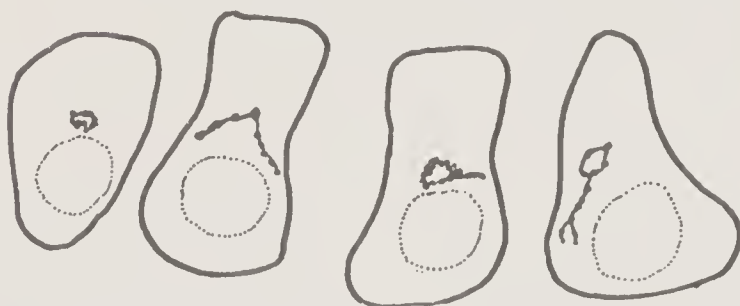


TEXT-FIG. 6. Neurone of developing chick. Golgi preparation.
Re-drawn after Alexenko³.

TEXT-FIG. 7. Vitamin C preparation of neurone of developing chick. Re-drawn after Barnett and Bourne¹⁴.

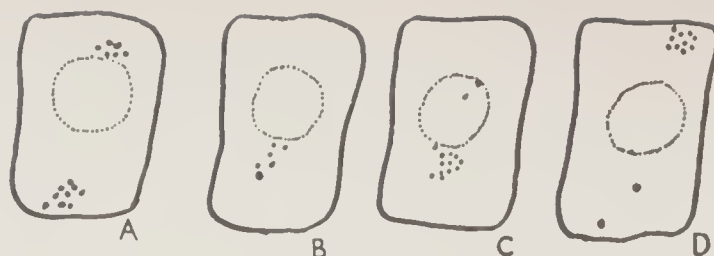


TEXT-FIG. 8. Golgi preparation of 11-day chick embryo liver.
Re-drawn after Dalton⁶³.

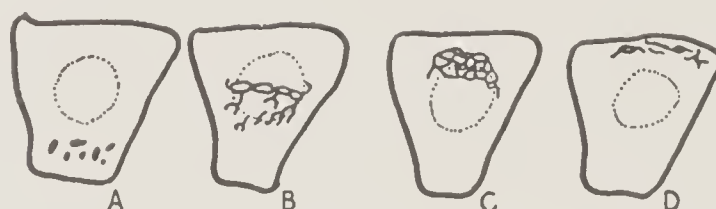


TEXT-FIG. 9. Vitamin C preparation of 10-day chick embryo liver.
Re-drawn after Barnett and Bourne¹⁴.

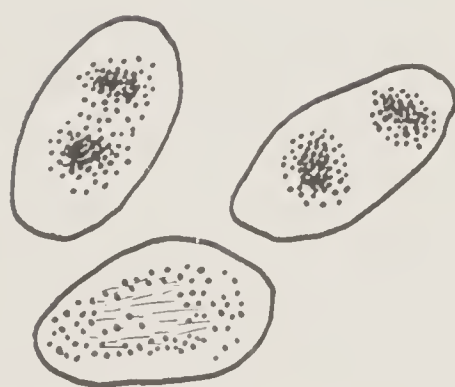
(demonstrated by standard techniques) in the damaged liver of rabbits is similar to the decrease in size shown by the area containing vitamin C (acetic acid/silver nitrate technique). There was a parallel increase in the Golgi apparatus and the vitamin C-staining area in the liver and capsule of the rabbit after injection of vitamin C. In scurvy in the marmot, Miwa showed a roughly parallel decrease in Golgi apparatus



TEXT-FIG. 10. Vitamin C preparation of 23-day chick metanephros, showing apparent passage of vitamin C across the cell.



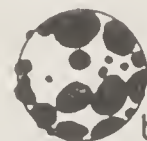
TEXT-FIG. 11. Changes in the position of the Golgi apparatus of the rat's kidney during diuresis. Re-drawn (modified) after Fischer⁷⁹.



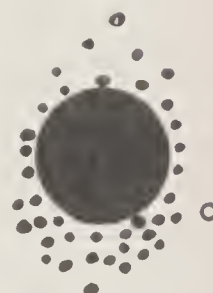
12



a



b



c

13

TEXT-FIG. 12. Golgi-like distribution of vitamin C granules in dividing cartilage cells of developing chick. Re-drawn after Barnett and Bourne¹⁴.

TEXT-FIG. 13. Stages in adsorption of vitamin C on to a 'Golgi system' in the intestinal cells of the starved *Ascaris*. Re-drawn after Hirsch¹⁰⁹.



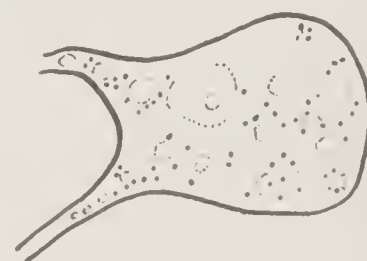
14



15



16



17

TEXT-FIG. 14. Vitamin C preparation of goblet cell of rat's ileum.

TEXT-FIG. 15. Golgi preparation of goblet cell of rat's colon. Re-drawn after Duthie⁷⁶.

TEXT-FIG. 16. Distribution of Golgi substance in fibroblast producing fat. Re-drawn after Macdougald and Gatenby¹⁵⁶.

TEXT-FIG. 17. Distribution of vitamin C granules in fibroblast producing fat.

and vitamin C-staining area in kidney and liver cells. Injection of vitamin C into a scorbutic marmot caused a parallel increase in Golgi apparatus and vitamin C-staining area in liver capsule and kidney cells. He concludes that vitamin C is associated with the Golgi apparatus.

In Hirsch's laboratory strips of intestine of the starved *Ascaris* were incubated in 0.1 per cent. solution of vitamin C. The cells absorbed the vitamin and concentrated it in the Golgi apparatus (Text-fig. 13). Hirsch suggested that the vitamin C was absorbed by the cells and distributed in the cytoplasm in a finely divided form which was not visible through a microscope, and that it was then concentrated in the Golgi apparatus.

Bourne (unpublished work) has obtained similar results with rats' intestines incubated with vitamin C in normal saline. Text-fig. 14 shows a goblet cell from a piece of ileum incubated in this fashion for half an hour and stained for vitamin C. It may be compared with Text-fig. 15, which shows a goblet cell of the rat's colon stained to demonstrate the Golgi apparatus.

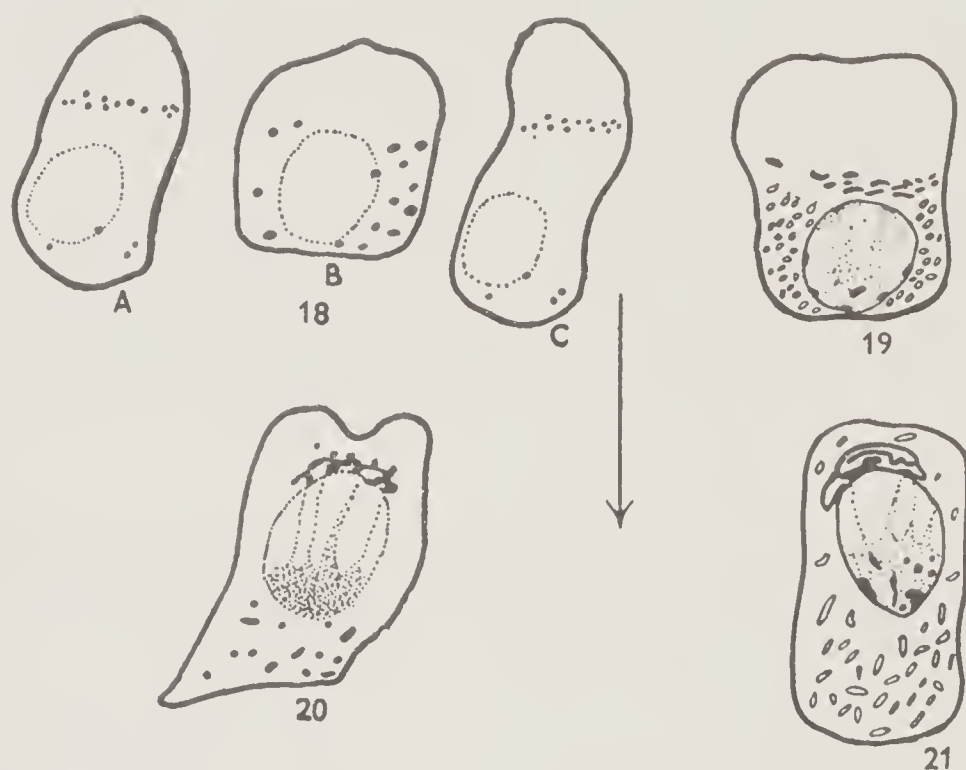
Hirsch¹⁰⁹ believes that the vitamin C first permeates the externum of the Golgi apparatus, but that finally it passes through it and becomes localized in the internum, where it plays a part in the formation of the 'product' of the Golgi apparatus (Text-fig. 28).

If fibroblasts in culture are stained with the silver nitrate reagent for vitamin C they give practically no reaction. Occasionally a few small granules may be found in the region of the idiozome. Cultured fibroblasts have, however, a well-defined Golgi apparatus (Plate 2, Fig. 3). When a fibroblast is forming fat the Golgi apparatus breaks up into small granules, which are scattered through the cytoplasm. These granules are often attached to the fat globules (Text-fig. 16). Vitamin C preparations of cells in this condition show the granules of vitamin C to be distributed in a similar fashion to the granules of Golgi material (Text-fig. 17). (Barnett and Bourne, unpublished work.)

Tonutti²¹⁴ has injected vitamin C together with trypan blue into animals and examined the kidney cells and, in the case of pregnant animals²¹⁶, the decidual cells. In each case the trypan blue was localized first in the region of the Golgi apparatus, and staining the tissue for vitamin C showed that in the centre of the blue areas produced by the trypan blue were black granules which indicated the presence of vitamin C (Plate 2, Fig. 10).

It was originally shown by Jasswoin¹²⁷ and more recently by Ludford¹⁴⁸ that trypan blue granules form in the region of the Golgi apparatus and then pass into the body of the cell in the same way as the secretion droplets of gland cells. Since Tonutti has shown that the droplets of dye so formed contain a central granule of vitamin C, it appears likely that the vitamin is adsorbed also on to the Golgi apparatus

and then segregated into granules in the same way as the trypan blue. It appears to be by means of granules such as these that vitamin C is passed across the kidney tubule cells. It has recently been shown by Fischer⁷⁹ (Text-fig. 11) that during artificially induced diuresis in the white mouse the Golgi apparatus of the tubule cells shows a periodical reversal of polarity. If this is so—for there is earlier work which denies



TEXT-FIG. 18 (A, B, and C). Ultracentrifuged cortical cells of rat's adrenal stained for vitamin C.

TEXT-FIG. 19. Ultracentrifuged cortical cell of rat's adrenal showing distribution of Golgi material (black rods) and mitochondria (ovals). After Dornfield⁷².

TEXT-FIG. 20. Ultracentrifuged medullary cell of rat's adrenal, stained to demonstrate vitamin C.

TEXT-FIG. 21. Ultracentrifuged medullary cell of rat's adrenal showing distribution of Golgi material (black network) and mitochondria (rods and ovals). After Dornfield⁷².

this change of polarity of the Golgi apparatus during diuresis (see Hirsch¹⁰⁹)—then it is possible that the Golgi apparatus itself acts as a transporting agent for vitamin C. The important point, however, is that the vitamin does not diffuse freely across the cell but is segregated by the Golgi apparatus, and makes its passage across the cell either in the apparatus or in the form of segregated granules.

The apparent passage of vitamin C through the tubule cells of the embryonic chick metanephros is shown in Text-fig. 10.

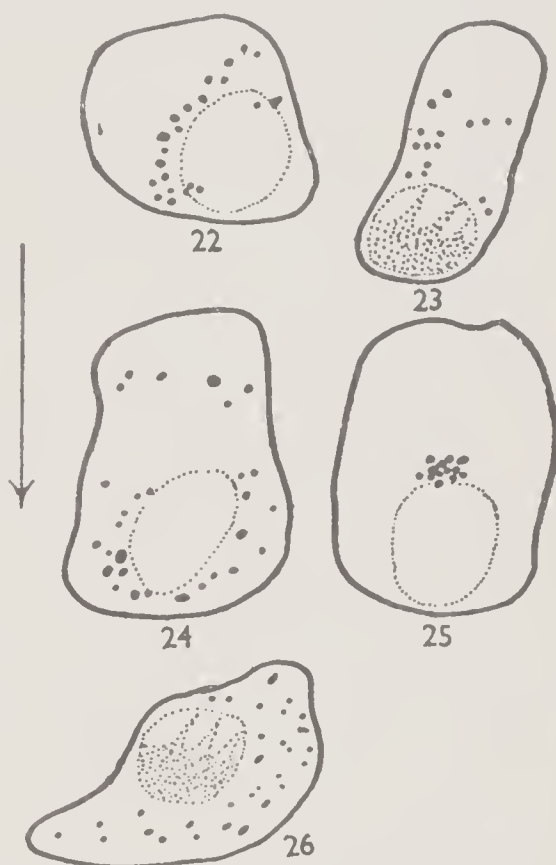
Baker¹¹ has shown that the vitamin C reaction appears to be localized in the osmiophil part of the Golgi element in oocytes and that it is shown, too, by the acrosome of newt spermatozoa.

When the adrenal gland of a rat is ultracentrifuged at a force of 400,000 g. for half an hour it has been shown (Dornfield^{72,73}) that the contents of the cells are stratified according to their relative densities.

For example, in the case of the cortical cell the nucleus is thrown centrifugally together with the mitochondria. The Golgi apparatus is situated above the nucleus (centripetal to it) and is often broken up into small granules (Text-fig. 19). In the medullary cell the nucleus is of different specific gravity relative to the cytoplasm and it is thrown centripetally, although the chromatin which it contains is thrown centrifugally, within the nuclear membrane. The Golgi apparatus is centripetal to the nucleus and the mitochondria are thrown centrifugally (Text-fig. 21). It is obvious that if such an ultracentrifuged adrenal is stained for vitamin C the disposition of the granules within the cell will provide an indication of whether mitochondria and Golgi apparatus contain the vitamin. Dr. J. St. L. Philpot has kindly ultracentrifuged the adrenal gland of a rat for the author for this purpose. The gland was ultracentrifuged at the speed producing a force of 300,000 g. for three-quarters of an hour. Microscopical examination after staining with the acetic acid/silver nitrate reagent showed three types of disposition of the granules. First, some cells in both cortex (Text-fig. 24) and medulla (Text-fig. 20 and Plate 3, Fig. 5) showed granules located in both the mitochondrial and Golgi regions; secondly, some showed the granules located principally in the Golgi region (Text-figs. 18 (A and C), 22, 23, 25; Plate 3, Figs. 6, 7, and 8); and thirdly, in others the granules were located principally in the mitochondrial region (Text-figs. 18 B and 26).

The specificity of the acid/silver nitrate method for vitamin C seems to be reasonably well established. It is important, however, to consider whether the localization of vitamin C in the Golgi region in some cells after the use of the acid/silver nitrate reagent represents an *in vivo* distribution of the vitamin.

Barnett and Fisher¹⁵ pointed out that in chemical models impregnated with vitamin C and then treated with acid/silver nitrate the black deposit of reduced silver appears to be localized at interfaces. Sosa (personal communication) believes that 'fixing' the tissue with hypo after



TEXT-FIGS. 22, 23, 24, 25. Various ultracentrifuged cortical cells of rat's adrenal stained to demonstrate vitamin C.

TEXT-FIG. 26. Ultracentrifuged medullary cell of rat's adrenal stained to demonstrate vitamin C.

treatment with acid/silver nitrate produces blackening in the Golgi apparatus. Deane and Morse⁶⁷ state that if more than three minutes elapsed between the death of the animal and placing of the tissue into silver nitrate there is an apparent concentration of the vitamin in the Golgi region, but not if it is placed in silver nitrate before that time. Pfuhl¹⁷⁸ considers that a substance as diffusible as vitamin C is not likely to be aggregated in life in a granular form similar to those found in the Golgi region of some cells. It is extremely unlikely, however, that vitamin C is in a highly diffusible condition in the cell, and in any case segregation is precisely what is likely to happen to a substance with as potent a reducing power as vitamin C, whether it is diffusible or not. In fact, the more diffusible it is, the more likely is it to be segregated in some organ such as the Golgi complex. (See also Bourne, *Nature*, in press.)

Nevertheless, serious consideration must be given to the possibility that the apparent localization of vitamin C in the Golgi region may be due to the adsorption on to its surface of micelles of reduced silver produced by the action of the vitamin in some other part of the cell. However, the evidence which is available suggests, in some types of cell at least, and in certain physiological states, that vitamin C is present in the Golgi region. Hoch-Ligeti and Bourne¹¹⁰ found that adrenal cortical cells showed a series of cycles in which vitamin C was first localized in the Golgi region; it was next seen in the form of a number of discrete granules aggregated at the cell membranes and could then be seen discharged into the surrounding blood sinusoids. The presence of such a cycle is of interest in view of the fact that it has been suggested that vitamin C may be attached to the cortical steroids on discharge into the blood-stream, as a solubilizing side chain. All the adrenals used for this work were placed in silver nitrate within three minutes of death and all were treated with hypo; that such diverse results as those described could be obtained suggests that the localization of the vitamin in the Golgi region is a function of the physiological activity and is not due to vagaries of the technique. A discussion of this problem of localization of the reaction is given by Bourne³¹. One may mention, however, that Silver¹⁹⁹ has shown that the Golgi region does not provide a surface which has a particular affinity for silver micelles and that it only does so after the cell has been treated with salts of heavy metals (e.g. uranium). It seems, therefore, that the localization of reduced silver in the Golgi region after the acid/silver nitrate technique is probably due not to the attraction of the region for silver micelles produced elsewhere in the cell but to the localization of the reducing substance there.

e. Alkaline Phosphatase and the Golgi Apparatus

It was first shown by Bourne³⁰ in 1943 that in some of the columnar epithelial cells of guinea-pig's jejunum alkaline phosphatase was

localized in the Golgi region. The localization of phosphatase in the same region of the cells of the mantle edge (which secretes the shell) of the mollusc *Mytilus* was also figured. Subsequent authors (Deane⁶⁵; Deane and Dempsey⁶⁶; Wislocki and Dempsey²³⁰; Emmel^{77,78}; and Al-Hussaini⁴) have also recorded the presence of this enzyme in the Golgi region.

In various mammals Deane and Dempsey⁶⁶ found that alkaline phosphatase was distributed in the Golgi region of the duodenal epithelial cells as granules or as a continuous reticulum. The reaction was most intense in the Golgi region of cells at the bases of the villi. Enzyme granules were also found in the Golgi region of cells of kidney tubules, bile capillaries, and uterine epithelial cells of various mammals. The same authors found acid phosphatase in the Golgi region of the duodenal cells (particularly in the mid-portion of villus) and in the uterine epithelial cells of pregnant cats and sows.

Deane and Dempsey went to some trouble to show that various methods of fixation did not affect the degree of localization of the enzyme into the Golgi region, and they concluded, therefore, that the reaction in this region represents a real concentration of enzymes during life. Emmel⁷⁷ found that after freezing-drying, the phosphatase in the Golgi region took the form of a discrete black area surrounded by a slightly diffuse boundary. Only rarely did he find a network. This is a form not unlike that shown in Baker's Sudan black and acid haematin preparations of the Golgi apparatus. After formol fixation the Golgi material had the form of a clear canalicular structure surrounded by a black deposit, indicating phosphatase activity. The canals he found to be devoid of phosphatase activity. Emmel also showed that KCN irreversibly inhibited phosphatase activity of the Golgi zones at a lower level of concentration than that at which it inhibits the phosphatase of the nucleus (nuclease?) and the cytoplasm. He believed, therefore, that the phosphatase of the Golgi zone was different from that in the rest of the cell.

Deane and Dempsey found that adenylic acid phosphatase (a pyrophosphatase) had a similar distribution in the different parts of the liver cells (e.g. appearing in the Golgi material of different cells at different pHs) as phosphatases hydrolysing glycerophosphate (phospho-monoesterase). It may be that the two separate phosphatases are located in the Golgi region or that a single phosphatase is able to hydrolyse the different substrates used. The same authors suggest that all cells may have significant phosphatase activity in the Golgi zone at some pH and with some substrate.

What is the function of the phosphatase in the Golgi apparatus? Emmel⁷⁷ points out that the enzyme is present in the lumen of intestine, kidney, uterus, and bile capillaries, and suggests that it is being

synthesized and excreted by the Golgi complex. Wislocki and Dempsey²³⁰ state that the formation of glycogen depends upon the dephosphorylation of hexose phosphate and the condensation of hexose molecules to form glycogen. Alkaline phosphatase might well be concerned in this process of dephosphorylation and so be concerned with the deposition of glycogen.

Although at present one can only guess at the functional relationship between the Golgi apparatus and alkaline phosphatase it is of great interest that this enzyme is concentrated in this region of the cell and that it is the first enzyme which has been so located.

f. Function of the Golgi Complex

The Golgi complex possesses the ability to adsorb various dyes. It seems able to absorb basic dyes *in vitro* and acid dyes only after injection. A basic dye, neutral red, is claimed by Hirsch to stain only the 'pre-substance' of the Golgi system. Chlopin⁴⁹ and others state that the neutral red forms new vacuoles in the cytoplasm. These vacuoles contain protein in solution and can therefore be fixed and stained. Chlopin calls these bodies the 'crinome'. Trypan blue, if it is injected (whereby it may be combined with a protein), becomes located exactly (according to Bowen³⁶) in the region of the Golgi apparatus of certain cells. Kedrowsky¹³³ has also found that if acid dyes are mixed with wheat-flour they can be adsorbed into the Golgi material of *Opalina*. This again suggests the attachment of an acid dye to a protein. In fact Hirsch states that the evidence available shows that acid dyes such as trypan blue can only be adsorbed on to the Golgi apparatus if they are combined with an albumen carrier. For an analytical review of vital staining see Ludford¹⁴⁹. Keller and Gicklehorn¹³⁴ have found that the majority of basic dyes, in the cell or in protein solutions, bear a negative electrical charge; the adsorption of basic dyes by the 'pre-substance' suggests that the latter bears a positive electrical charge. Hirsch states also that there is evidence that the Golgi system itself is positively charged. The fact that the apparatus in some cells adsorbs vitamin C, which probably carries a negative charge, supports this view. The fact that in the same cell types on different occasions the mitochondria adsorb vitamin C and on others the Golgi apparatus does so may suggest a varying charge on the two structures.

Hirsch and Bretschneider¹⁰⁹ have found that if iron sugar is fed to an animal the iron is adsorbed on to the Golgi apparatus and not on to the mitochondria or the nucleus (Plate 4, Fig. 1). Kedrowsky^{132,133}, however, found that iron could only be adsorbed by the Golgi bodies of *Opalina* if it was mixed with peptone. It is of interest that a substance known as iron ascorbate (iron salt of vitamin C) exists and may have a physiological function; the fact that iron and vitamin C both aggregate

at the Golgi apparatus suggests the sort of function the apparatus may have, namely, that of bringing various reactants into contact with each other.

Van Teel²¹⁸ has shown that Golgi systems also adsorb compounds of copper and gold. Silver compounds are also localized in this region of the cell, and Kedrowsky¹³² has shown that the Golgi bodies of *Opalina* can adsorb bismutose (a compound of albumen and bismuth) and protargol (a compound of albumen and silver).

The adsorptive properties of the Golgi apparatus mentioned above lend colour to the speculation that the Golgi apparatus acts as a condensation membrane. Of this theory Kirkman and Severinghaus¹³⁸ state:

A great deal of work strongly suggests that the Golgi apparatus neither synthesises secretory substances nor is transformed directly into them; but it acts as a condensation membrane for the concentration, into droplets or granules, of products elaborated elsewhere and diffused into the cytoplasm. These elaborated products may be lipoids, yolk, bile constituents, enzymes, hormones, or almost any other formed substance.

The conception of the Golgi apparatus purely as a condensation membrane may be only half the truth, for it seems possible that synthetic products may also be formed in the interior of the apparatus. Bowen speaks of the Golgi apparatus as 'a great intracellular centre of chemical synthesis or enzyme formation'. Kirkman and Severinghaus lay considerable stress on the condensation membrane hypothesis, and point out that since the Golgi apparatus segregates acid dyestuffs into droplets 'one must again conclude that the topographical appearance of secretory droplets, as evinced in the reaction of kidney and liver cells to acid vital dyes, suggests nothing so much as a segregating and condensing function for the Golgi substances'.

Earlier workers believed there was an association between the Golgi apparatus and secretion, and this was put forward more definitely by Nasonow¹⁷¹ in 1923. Bowen's brilliant series of investigations proved conclusively the association of the Golgi apparatus with the production of secretion droplets in various types of exocrine gland. Droplets of secretion appear in the interstices of the Golgi network, and as they grow in number so the apparatus hypertrophies, and finally, when the cell carries its full load of secretion, the apparatus appears to break up and small pieces become attached to some of the secretory granules (Plate 4, Figs. 4 and 7). Bowen worked with the well-known externally secreting glands. There is evidence also that the apparatus is associated with the secretion of bile by the liver, of synovial fluid by the synovial membrane, in the production of the enamel of teeth by ameloblasts⁹ (Plate 4, Fig. 2), the production of pigment by the cells of the iris epithelium, the formation of fatty yolk in eggs, and of the acrosome in spermatozoa. Hypertrophy of the apparatus is also found in cells which

are undergoing cornification and in connective tissue which is forming fat, and in the formation of cartilage. Cramer and Ludford⁶² state that the apparatus is actively concerned in the process of resynthesis of fats from the fatty acids and glycerol absorbed by the intestinal cells.

Moricard¹⁷⁰ has shown that the Golgi complex is associated with the production of follicular fluid by the granulosa cells of the ovary. Järvi^{124,125,126} has shown that in the pancreas pigmented bodies appear to be produced in this region, and Sluiter^{200,201} has shown that in the same organ the pro-enzyme granules appear to have a functional relationship with the Golgi complex.

Wimsatt²²⁹ says that the Golgi 'end' of the cells in the endometrium of the bat are metabolically more active than the opposite end of the cell. He points out that absorbed materials must pass through the Golgi region which has, therefore, an opportunity of acting on these materials. Wimsatt also found considerable differences in the form and distribution of the Golgi material of the mesenchymal and trophoblast cells of the bat placenta at different stages of pregnancy. Wislocki and co-workers found that in the human syncytial trophoblast the Golgi substance was usually in a granular dispersed form, and they thought that this might be related to the need for equal distribution of the Golgi substance throughout growing tissues.

Aykroyd and Gatenby⁹ found that changes in the Golgi material of human uterine gland cells could be correlated with functional activity.

There is some evidence that the Golgi material may be concerned with the elaboration of endocrine products or with the direction in which the products are secreted; e.g. Cowdry has suggested that the reversed polarity of the Golgi apparatus which is seen in some thyroid cells is an indication of reversal of secretion; that the cell, instead of discharging into the lumen, discharges directly into the blood-stream. This reversal of polarity as an indication of reversal of secretion is not upheld by all other workers. (For a discussion of the Golgi apparatus and thyroid secretion see Chapter 10.)

Vasquez-Lopez²¹⁹ has suggested that the changes in the Golgi material of the pancreatic islet cells give an indication of secretion.

In the parathyroid gland Rosof¹⁹⁵ has suggested that the Golgi material varies in form with the secretory cycle, a conclusion which has been confirmed by Foster⁸¹. De Robertis⁷¹ found that injection of parathyroid extract into an animal caused considerable changes in the form of the Golgi apparatus which continued for some 15 days after the injection.

Courrier and Reiss⁵⁶ stated that the cells of the parathyroid gland were arranged in cylindrical columns, that these columns were drained by a central capillary and supplied by nutritive capillaries. The authors claimed that the Golgi apparatus of the parathyroid cells was orientated

between the cell nucleus and the central vein. Other workers, however, have found that many parathyroid cells do not show this orientation of the Golgi apparatus.

Several workers have attempted to correlate changes in the Golgi apparatus of cells of the anterior pituitary gland with functional changes in the gland or with the presence or absence of granules in the cells. Atwell⁷, for example, found no hypertrophy of Golgi material in fully granulated cells; a finding supported by Foster⁸³. Haquist¹⁰¹ found that in the female guinea-pig a loss of granules (both eosinophil and basophil cells) could be associated with a progressive hypertrophy of the Golgi material. Ayer⁸, describing vesicles moving out from the Golgi region in the hypophysis of the young rat, suggested this may have been the origin of the endocrine secretions of the cells.

Reiss¹⁸⁸ stated that in the hypophysis of the cat the chromophobe cells showed no particular orientation of the apparatus, suggesting that these cells were in a resting condition. In the acidophilic cells the Golgi apparatus occurred between the nucleus and the central area. In basophils it was placed peripherally. Reiss stated that all stages of transition between the two types of cells could be observed, and he expressed his belief that the cell can pour out a secretion at the periphery and then reverse and discharge a different substance into the centre of the cluster. Severinghaus found, however, that the Golgi apparatus was not orientated towards the lumen of the blood capillaries in either basophils or acidophils. He claims that there is no justification for using its position in the cell as an indicator of secretory activity. Severinghaus¹⁹⁸ also found two distinct types of Golgi apparatus in the chromophobes; one type of apparatus was comparable with that found in acidophils and the other with that found in basophils. He believes that this is evidence that these cells arise separately from the chromophobes and that they are not actually interchangeable.

Foster⁸³ studied the Golgi material in the anterior pituitary gland of the rat, using Sudan black. He found the appearance of the material was substantially the same as that obtained by the more usual Golgi techniques. The Golgi zone he found to contain diffuse sudanophil material in which were embedded clear vacuoles and strongly sudanophil bodies, the last being often associated with the vacuoles as caps or rims or granules. Administration of stilboestrol to the animal he found to cause an increase in the number of clear vacuoles. He found a more variable increase in the amount of strongly sudanophil material.

Reese and Moon¹⁸⁷ found a hypertrophy of the Golgi material in the adrenal cortical cells of the cat after injection of adreno-cortico-trophic hormone, but Bennett²¹ was not able to correlate changes in the Golgi material with secretion in the cat adrenal. Bourne²⁷ attempted to demonstrate that the Golgi material in the adrenal cortical cells was

concerned with the production of lipid droplets. No unequivocal evidence that this was so could be obtained, but it was found that in cells containing few or no lipid droplets in the secretory part of the cortex the Golgi apparatus was invariably small and compact. On the other hand, cells which were full of lipid droplets could be divided into two types, those with a small apparatus and those with a hypertrophied and partly fragmented apparatus. It appeared that at some stage the Golgi material might play a part in the maturation of the lipid droplets. Types of Golgi distribution present in adrenal cortex and medulla can be seen in Plate 3, Fig. 1, A-H. These may be compared with the vitamin C preparations of adrenal cells in Plate 3, Figs. 2, 3, 4. The similarity of the Golgi preparations to vitamin C preparations suggests that the spreading of the Golgi material through the lipid droplets may be concerned with the linking of vitamin C on to the sterol molecule, a process which is believed to occur at some stage before secretion of the cortical steroids.

A review of the Golgi apparatus and endocrine secretion is given by Foster⁸⁰.

McManus^{163,164,165} has recorded an interesting change of polarity of the Golgi complex in the first and second convoluted tubules of the kidney. In these tubules the Golgi material is on the side of the nucleus adjacent to the tubular lumen, but in the *macula densa* the cells of the second convoluted tubule adjacent to the glomerulus show the Golgi material on the other side of the nucleus. He suggests a functional explanation for this:

1. That some substance is passing across the cells of the tubules to the specialized cells of the glomerular root.
2. That this process is the method by means of which the haemodynamics of the nephron are integrated.

This reversal of polarity was found in the kidneys of cat, rabbit, and human.

It has been said that because a cell organ such as the Golgi apparatus hypertrophies it is not necessarily bound up with the secretion of certain droplets which are seen near it or in its interstices. It may be true that the actual substance of the Golgi apparatus may not be modified into secretory droplets, but the syntheses of the cell take place as a result of the catalytic action of surfaces, and the hypertrophy and partial fragmentation of the Golgi apparatus are undoubtedly the result of the necessity within the cell for an increase of synthetic surface. It is possible, too, that enzymes are adsorbed on to the surface of the apparatus. The same argument applies to mitochondria. When they are filamentous they are providing the smallest, and when they are in granules they are providing the greatest possible surface. One reason why the surfaces of a cell are so important, and why they are probably

the seat of syntheses, is that surfaces are invariably regions of free energy. Synthesis requires energy, and if the chemical reactions of the cell are to obey the Second Law of Thermodynamics, such syntheses must take place where free energy is available. Oxidative reactions occur most actively in the living cell in contact with solid or structural cell material, i.e. at the boundary of protoplasmic phases—in other words, at the surfaces of membranes, fibrils, granules, and other solid cell structures, which appear to have a catalytic or accelerating influence on these reactions. This has been tested with living cells by the use of reagents which become coloured on oxidation. Lillie¹⁴⁴ says that in areas of the cell surrounded by membranes a 'variety of physiological or metabolic activities may proceed side by side in the same cell without interfering with one another'. Lillie quotes the Golgi apparatus and the mitochondria as two examples of such areas. It may be significant that these organelles are often associated with other cell membranes; e.g. the mitochondria are often aggregated near the nucleus or the cell membrane, and the Golgi apparatus is often associated with the nuclear membrane.

It is known that the velocity of any reaction taking place at a surface will be greater the larger the surface exposed. In colloid systems there exists what Alexander² calls a zone of maximum colloidity, which means that in such a system too great a degree of aggregation results in only a relatively small surface area being available and a given reaction proceeds too slowly; in a condition of too great a dispersal the reaction proceeds too rapidly. Therefore, to obtain the appropriate speed there must be a balance between the two states of dispersal. Once again we may apply this analogy to the mitochondria and the Golgi apparatus. There may be a mitochondrial/Golgi-surface to cell-volume ratio for which the cell has its optimum function. An alteration of this surface: volume ratio by bacterial toxins, undue amounts of hormones or drugs, or deficiency of minerals or vitamins may be one of the factors involved in the state known as ill health. Examples are the formation of large mitochondrial masses in scurvy (Bourne²⁸) and the increase in the amount of Golgi material in the nerve cells in beri-beri (Chang⁴⁸). Also, Horning has investigated the effects of acute and chronic morphine poisoning in the Golgi apparatus of various tissues of the white rat. In the spinal neurones of animals with acute morphine poisoning (as a result of massive injections of morphine) he found that there was a hypertrophy and fragmentation of the Golgi material. These changes are beautifully illustrated in Plates 5 and 6, the photomicrographs for which were kindly supplied by Dr. Horning.

In experimental pneumonia Tonutti²¹⁴ has found that alveolar phagocytes mobilized in the lung contained a large number of vitamin C granules. Since vitamin C is usually segregated in either the Golgi

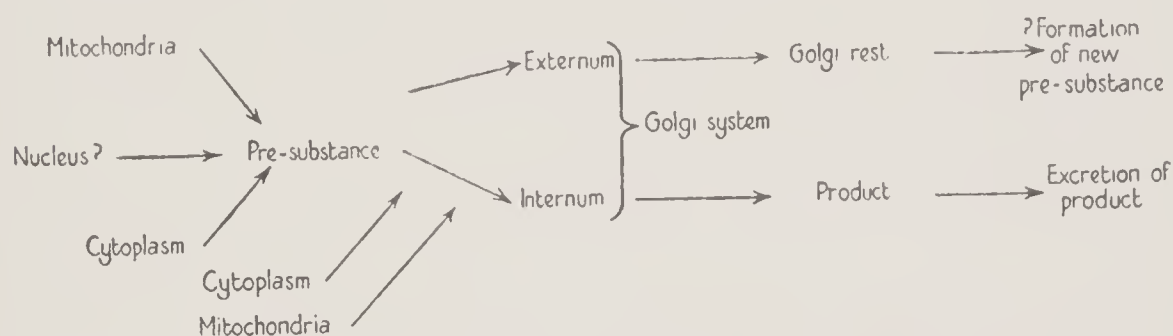
apparatus or in the mitochondria, this suggests that one or the other of these organelles has broken up into innumerable granules and has thus enormously increased the amount of surface available (Plate 1, Fig. 6). It has been shown by Martinson and Fettisenko^{160,161,162} that vitamin C stimulates both lipolysis and proteolysis, and therefore such a cell is well equipped to deal with phagocytosed material.

Bayliss¹⁶ has described the living cell as a heterogeneous system. In a homogeneous system all the molecules are equally accessible to external influences, either chemical or physical, and in heterogeneous systems only those at the surface of each phase are so accessible. Therefore, only the superficial molecules of the mitochondria or Golgi apparatus are capable of direct reaction with the cytoplasm. Although we cannot conceive of mitochondrial or Golgi apparatus activity apart from the cytoplasm in which it lies, we must remember that some substances, e.g. vitamin C, can pass into the interior of these organs and that, therefore, reactions can proceed inside both the mitochondria and the Golgi apparatus which would be quite different from those proceeding at the surface. It seems that both Golgi apparatus and mitochondria are in a state of chemical balance, and that changes in the permeability of the cell membrane or of their own membranes may swing their activity from rest to synthesis or lysis.

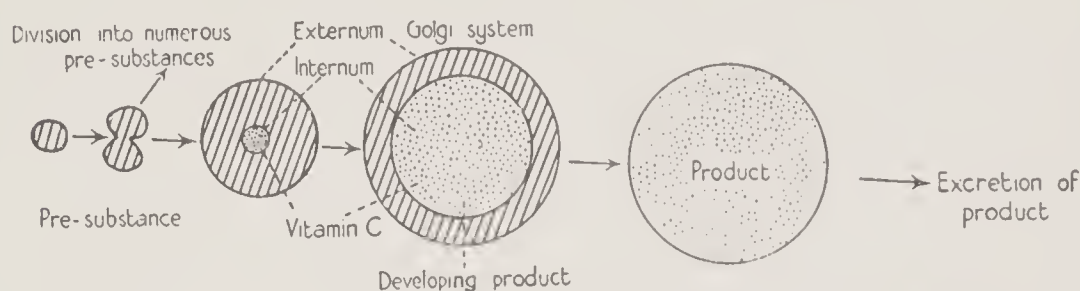
The Golgi apparatus appears to be intimately associated with cellular synthesis, and it has been known for some time that in scurvy synthetic processes of cells are inhibited. Hirsch claims that when a cell is about to form a 'product' the Golgi apparatus adsorbs vitamin C. He says that prior to the formation of secretion droplets in pancreas cells, vitamin C can be demonstrated in the Golgi region. Klein¹³⁹ has shown that osteoblasts may also contain aggregations of vitamin C in the same region of the cell. As mentioned before, fibroblasts *in vitro* show the presence of vitamin C in the scattered Golgi material only when the cell is producing fat. Cartilage cells in the chick embryo, prior to the hypertrophy which precedes bone formation, show dense aggregations of granules, not only in the Golgi apparatus, but packing the rest of the cytoplasm as well. Also, in the histogenesis of other organs in the chick embryo, particularly the liver and the brain, the cells show (as has already been mentioned) an aggregation of vitamin C granules in those regions of the cell where one would normally expect a Golgi apparatus to be situated. In healing wounds (Bourne, unpublished work), cells are present which contain abundant black granules, many of them aggregated near the nucleus in what appears to be the Golgi region of the cell, but others are lying in the body of the cytoplasm; these cells are probably macrophages and may be assumed to be producing enzymes for the intracellular digestion of engulfed material. It is noteworthy, too, that the outer cells of the zona fasciculata of the adrenal gland

show vitamin C granules aggregated in the Golgi region, whereas this is not always so in the inner cells of this zone. Bennett²¹ has shown that it is the outer cells of the zona fasciculata which contain the biologically active 17-keto steroids.

These facts lend strong support to the conception that vitamin C, through its association with the Golgi system, plays a part in a wide



TEXT-FIG. 27. Relationship of the Golgi apparatus to other parts of the cell and to the formation of a product. Re-drawn after Hirsch¹⁰⁹.



TEXT-FIG. 28. Relationship of vitamin C to the Golgi apparatus. Re-drawn after Hirsch¹⁰⁹.

variety of cellular syntheses, but it seems unlikely that the vitamin plays a direct part in the synthesis of such different chemical substances. Tonutti thinks that the Golgi apparatus stores vitamin C and liberates it slowly into the cytoplasm in sufficient amounts to prevent oxidation of the cell products.

A more likely explanation seems to be (since the metabolism of cells is raised when they are carrying out synthetic processes) that unless these various products are being protected by being produced in, or adsorbed on to, a specially segregated, highly reducing area of the cytoplasm, they would be oxidized as rapidly as they are formed.

Wolf-Heidegger²³² has suggested a converse relationship between vitamin C and the Golgi apparatus in the cells of the teli of the choroid plexus. He claims that the vitamin enters the cerebro-spinal fluid from the blood in this region. In the blood the vitamin is chiefly in the reversibly oxidized form, and in the cerebro-spinal fluid it is in the reduced condition. During the passage of the vitamin across the cells of the choroid plexus, reduction must occur. Wolf-Heidegger states that the vitamin is adsorbed on to the Golgi apparatus and he believes that this is the region where reduction takes place. If this is so it is possible that

the second reducing substance may be glutathione which, incidentally, does not react with acetic acid/silver nitrate. There is a roughly parallel distribution of glutathione and vitamin C in the body, and the two substances are known to be capable of forming an oxidation-reduction system. It should be borne in mind that the Golgi apparatus in cells other than those of the choroid plexus may be the locus of a 'redox' system based on vitamin C and glutathione. Järvi¹²⁶ believes that vitamin C, through its association with the Golgi region in pancreatic cells, is concerned with the production of zymogen granules in that organ. This, however, is denied by Sluiter²⁰¹.

One can conceive of the function of the Golgi apparatus in gland cells, but it is difficult to understand its function in non-secretory cells such as the nerve cell. Some workers have claimed that its function in the nerve cells of *Helix* is to secrete fat. A more likely activity is suggested by Brambell³⁸, based on his work on *Helix*. He believes that in the neurones the apparatus is responsible for the secretion of the Nissl granules. He found Nissl granules distributed in the same manner as the Golgi apparatus, and Nissl granules were most marked in those cells in which the apparatus is very active. Andrew⁶, in the Purkinje cells of young and adult mice, found distinct Golgi nets, but in senile animals he found the Golgi material in the form of scattered granules.

Cajal⁴⁶ and Penfield¹⁷⁶ found a fragmentation and regression of the Golgi apparatus of spinal neurones following section of their axones.

The Golgi apparatus increases in amount in beri-beri (Plate 4, Figs. 5 and 6). This gives the impression that it is undergoing a compensatory hypertrophy. If this is so it is of special interest because of the chemical changes found in nervous tissue in beri-beri by Peters and his co-workers.¹⁷⁷ It is tempting, therefore, to postulate that the Golgi apparatus is associated with carbohydrate metabolism.

Although there are indications of the function which the Golgi complex may have we are still in the position of having very little certain knowledge of its true role in cell physiology.

Nevertheless, it seems safe to assume that the Golgi region is one which is capable of the segregation of dyes and other substances and that this property serves two purposes. Firstly, it has a protective action both for the products of cellular activity, protecting them from being further metabolized after they are formed, and for the cell itself, by segregating toxic substances and substances such as vitamin C, which would seriously interfere with the normal activity of the cell if they were diffused through the cytoplasm. Secondly, it causes aggregation of products found in the rest of the cytoplasm into secretion droplets ready for excretion.

A possible further function is that of bringing reactants into contact with each other by their adsorption on to this region of the cell. That

the substance of the Golgi complex itself takes part in reactions in this region, e.g. the phosphatase in the Golgi region of intestinal cells, is likely.

What chemical part the Golgi complex plays in the physiology of the cell we cannot say until we know for certain to what extent enzymes, vitamins, and so on are concentrated in it, but slowly we seem to be building up a picture of the physical part it plays in the cells' activities.

g. Summary of the Golgi Complex

The so-called Golgi apparatus is probably more correctly described as the Golgi complex, since it has been shown to consist, in some cells at least, of three types of material, i.e. spheroids with a lipoidal cortex, dense lipoid, and diffuse lipoid.

This structure explains the variety of forms which the Golgi complex can apparently assume. It is probable that the complex is constantly changing its form in individual cells, although cells of the same organ appear to have a Golgi complex of similar general pattern.

The presence of spheroids in a lipoid matrix which diffuses into the surrounding cytoplasm without definable membranes would explain most of the observed facts concerning the staining and physical manifestations of this enigmatical region of the cell.

It is certain that the Golgi complex in all cells does not have this triple structure, and in some invertebrate cells the spheroids do not appear to be embedded in lipoidal material; while in some vertebrate cells photographs taken by ultraviolet light seem to show that the Golgi region is bounded by a membrane (see Chapter 10).

The Golgi region in some cells has been shown to contain lecithin, cephalin, or sphingomyelin. Carotene has also been found to be present in one case. It also contains protein, but not more than the cytoplasm. Vitamin C and alkaline and acid phosphatases have also been shown to be present in this region of some cells.

The Golgi complex is certainly concerned in the production of the secretion of exocrine glands and it may be associated with the formation of endocrine products.

It acts as a condensation membrane, segregating substances to be secreted and potentially toxic substances. It can act, therefore, as a protective mechanism for the cell and as a synthetic centre.

It probably plays a direct chemical part in some cellular syntheses.

It appears to play some part in the differentiation of embryonic cells, for it is active during histogenesis.

Addendum: While this book was in the press, papers by Pallade and Claude (*J. Morphol.* 1949, 85, 35, 71) have suggested that the conventional Golgi net is an artefact composed of myelin figures produced by the action of fixatives on the lipoid containing neutral-red staining spheroids present in the Golgi region of the cell.

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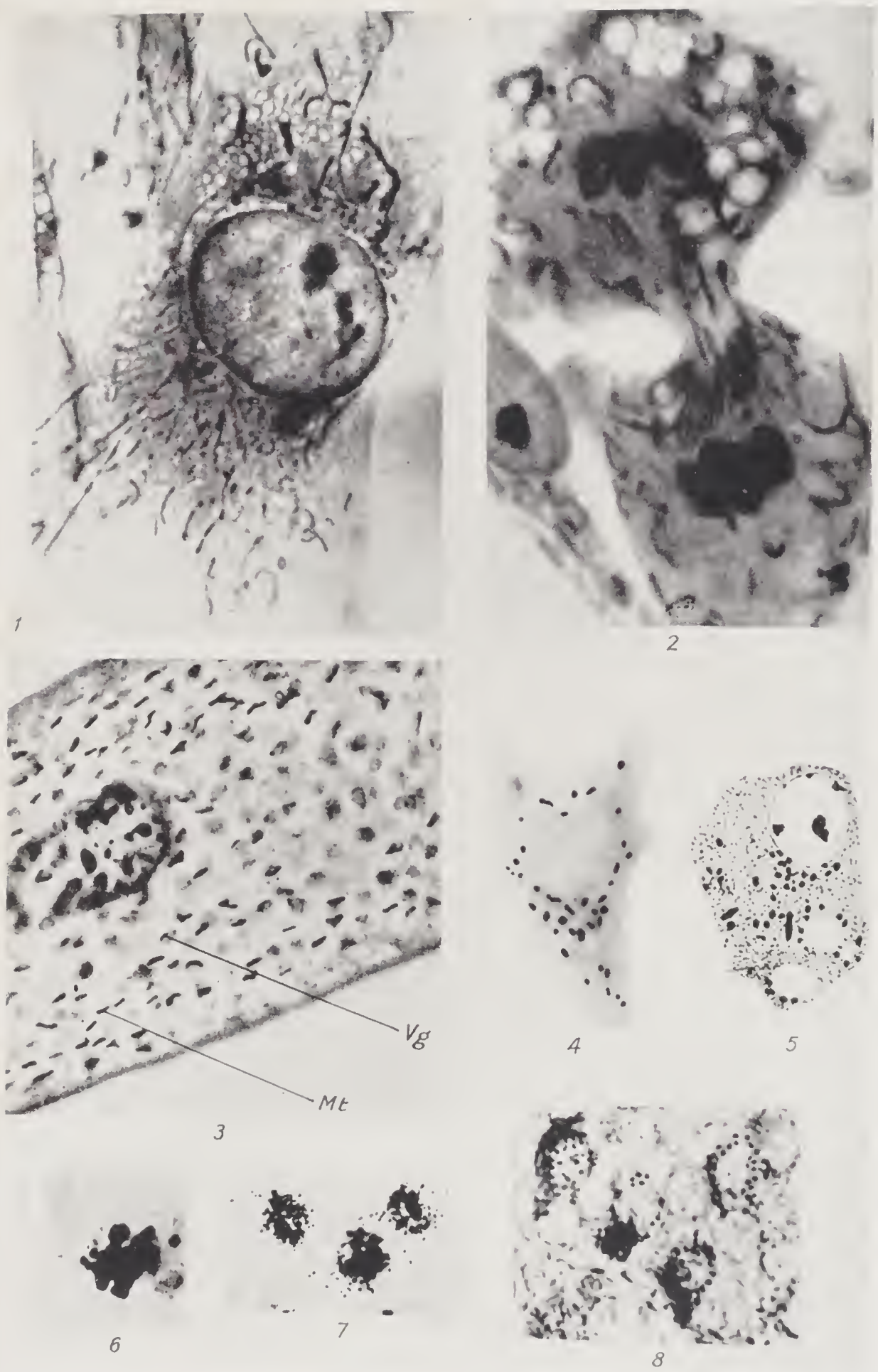
DESCRIPTION OF PLATES

PLATE 1

FIG. 1. Myoblast from culture of ventricle of 6-day chick embryo. The mitochondria may be seen as rods and long filaments. From Richardson (1934).

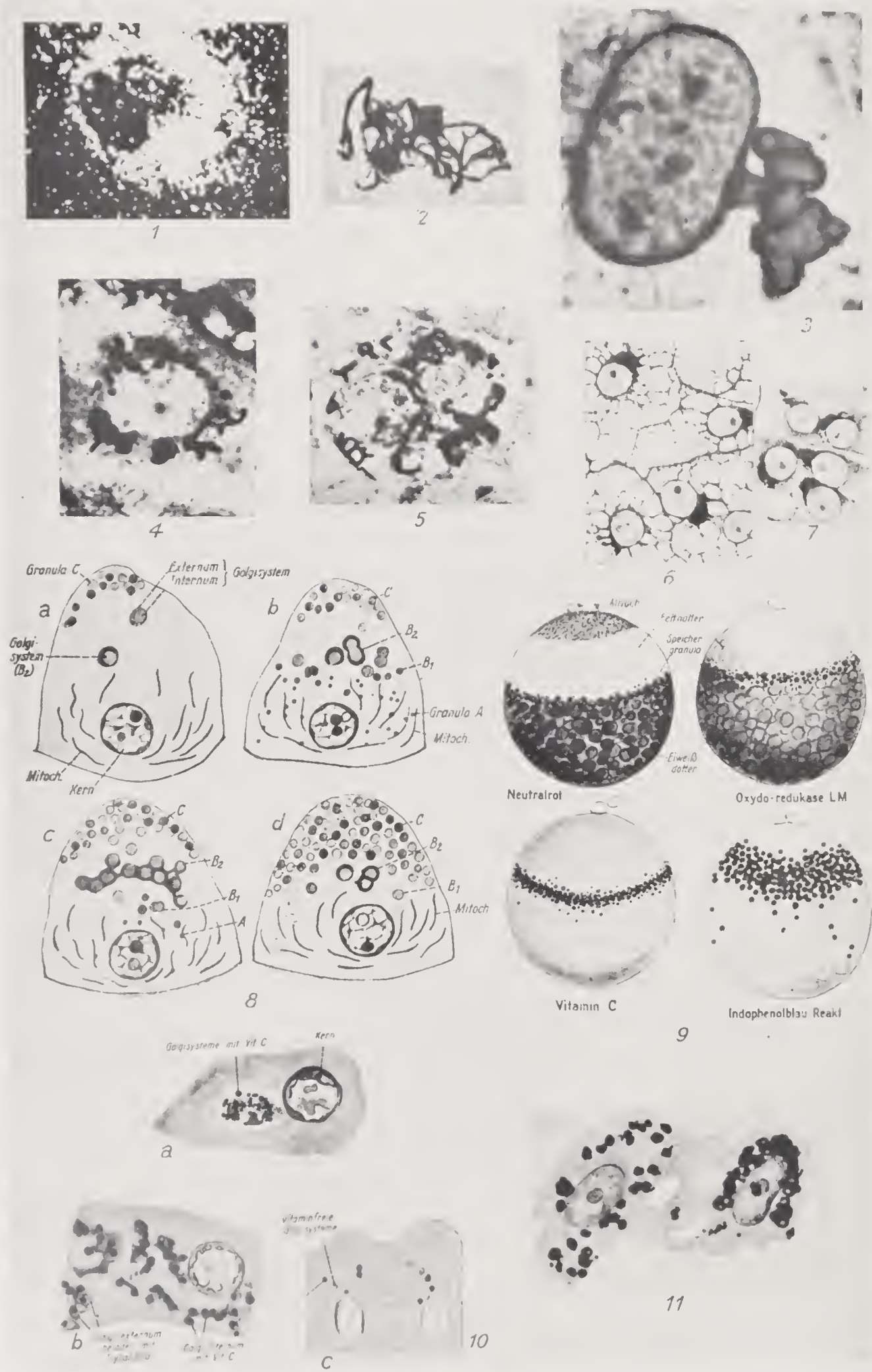
FIG. 2. Telophase in myoblast from culture of ventricle of 6-day chick embryo. The mitochondria are distributed more or less equally between the two daughter-cells. From Richardson (1934).

FIG. 3. Section of *Opalina*. The mitochondria (Mt) may be seen as small rods associated with grey spheres—the vegetative granules (Vg). From Richardson and Horning (1931).



Mitochondria and vitamin C in various cells

G. H. Bourne



Golgi apparatus and vitamin C in various cells

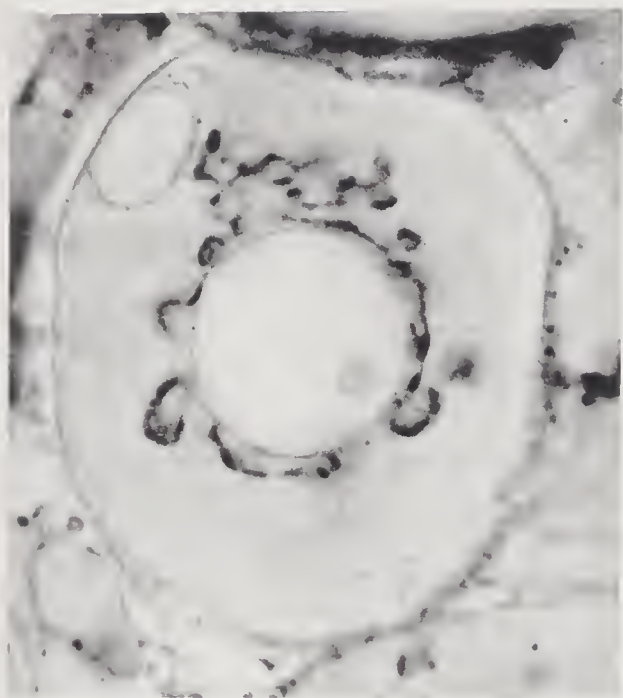


Golgi apparatus and vitamin C in adrenal cells

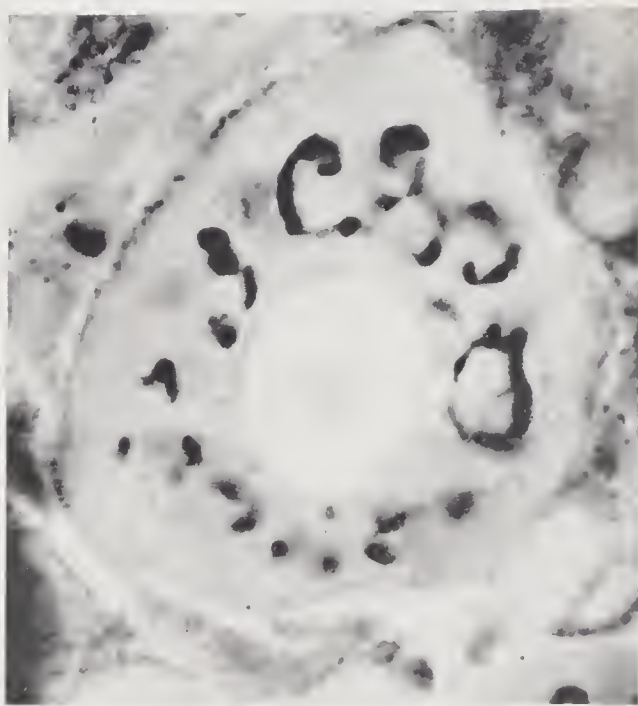
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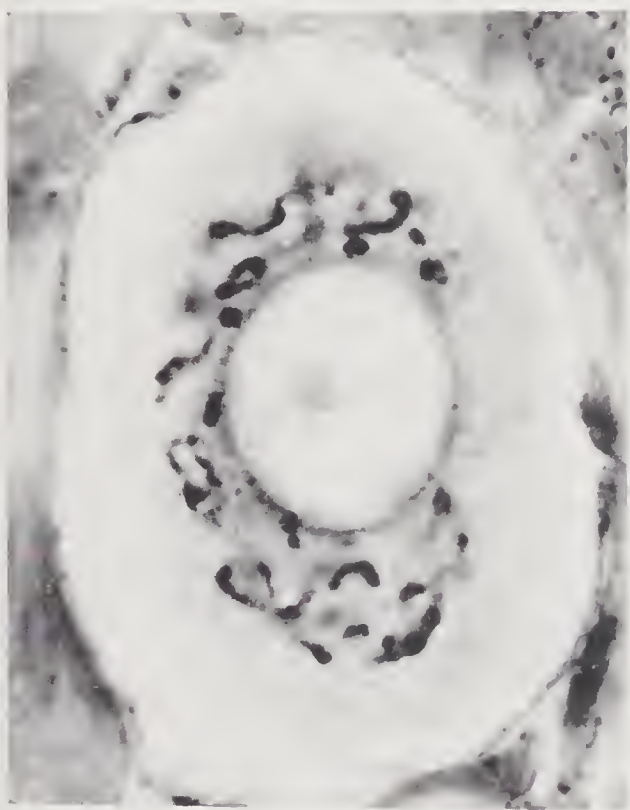
Golgi apparatus in various cells



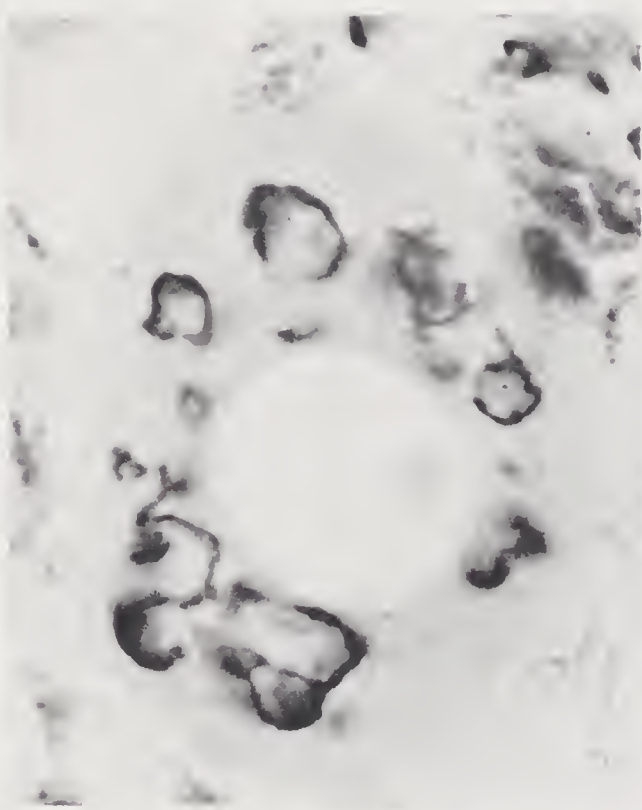
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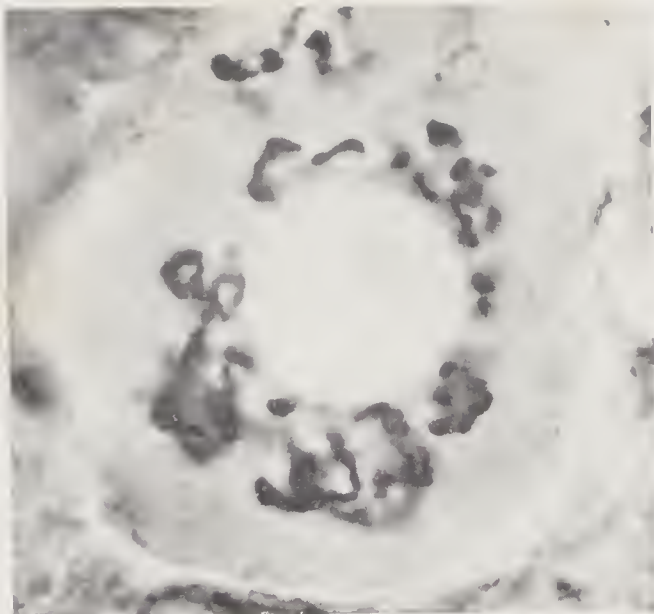
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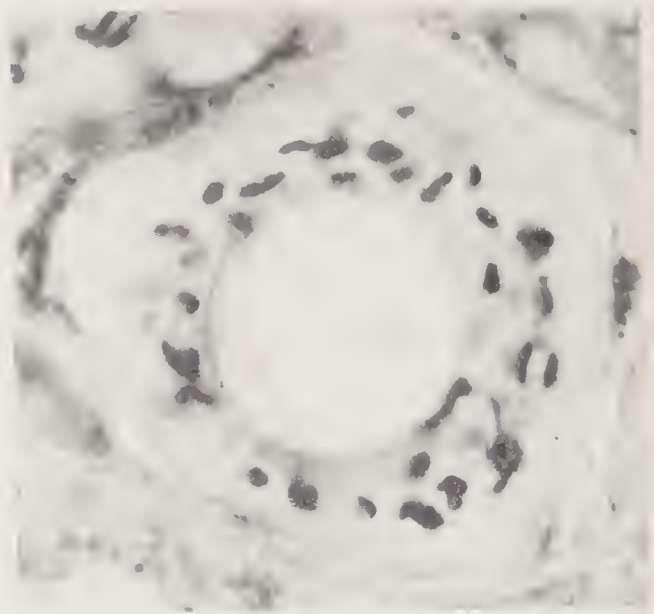
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Golgi apparatus in morphine poisoning

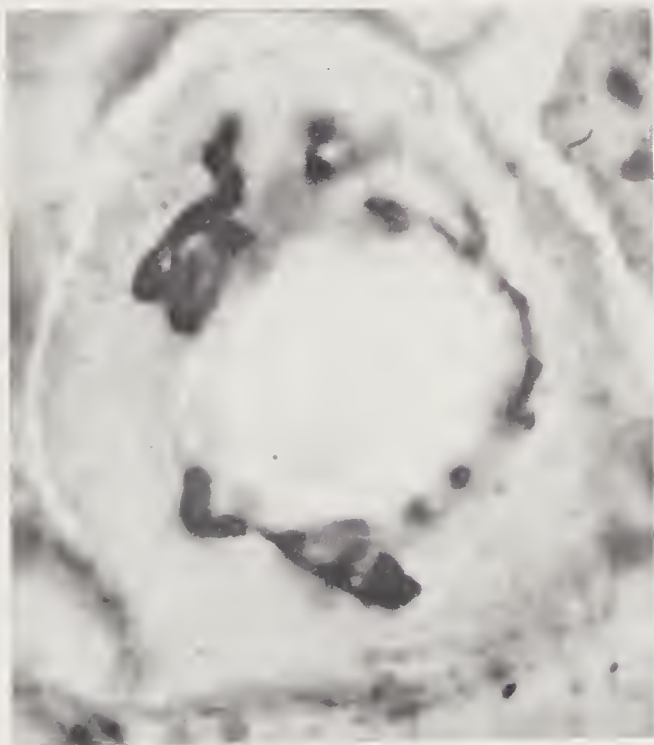
G. H. Bourne



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Golgi apparatus in morphine poisoning

FIG. 4. Vitamin C preparation of cell from adrenal medulla of rat. The granules have a mitochondrial appearance. (Re-touched.) $\times 1,200$. Compare with Fig. 5.

FIG. 5. Mitochondrial preparation of cell from adrenal medulla of guinea-pig. From Mulon, after Rogoff and Stewart (1932).

FIG. 6. Histiocyte from infected lung. Stained to demonstrate vitamin C. The entire cell may be seen to be packed with granules. From Tonutti (1938).

FIG. 7. Vitamin C preparation of corpus luteum cells, showing mitochondrial appearance of granules. After Giroud (1938).

FIG. 8. Vitamin C preparation of anterior pituitary gland of rat, showing mitochondrial appearance of granules in chromophilic cells. The chromophobe cells give no reaction with the silver nitrate reagent. (Re-touched.)

PLATE 2

FIG. 1. Living fibroblast from culture of rat heart. Dark ground illumination. The nucleus may be seen as a black oval mass near left border of light area represented by the cell. Immediately to the right of the nucleus and in contact with it is an irregular black area speckled with white. This is the Golgi apparatus. From Ludford (1935).

FIG. 2. Electron microscope photograph of an apparently homogeneous silver granule in a photographic emulsion. (Magnification 25,000 diameters. By permission of Science Service, Washington, and the Research Laboratories of Kodak Ltd.)

FIG. 3. Epithelial cell from culture of chick liver. The Golgi apparatus may be seen to the right of the nucleus. The division into inner and outer portions may easily be seen. From Richardson (1934).

FIG. 4. Hepatic cell of mouse. Kolatchev preparation. The Golgi apparatus may be seen surrounding the nucleus. (Re-touched.) From Tarao (1939).

FIG. 5. Hepatic cells of mouse digested by pepsin and stained with Nile blue sulphate. Two nuclei are visible, and the stained material may be seen to occupy a similar position to the Golgi apparatus demonstrated by Kolatchev's method in Fig. 4. (Re-touched.) From Tarao (1939).

FIG. 6. Golgi preparation of cells from secretory zone of adrenal cortex of cat. From Bennett (1940).

FIG. 7. Golgi preparations of zona glomerulosa cells of adrenal of cat. From Bennett (1940).

FIG. 8. Stages in secretion of zymogen granules in the pancreas. (For details see text.) The second cell shows the passage of small droplets from the mitochondria to the Golgi field. From Hirsch (1939).

FIG. 9. The staining of the egg of *Aplysia* by various techniques. The *Speicher granula* may represent the Golgi material. It may be seen that these granules react with neutral red and appear to contain vitamin C. From Ries (1937).

FIG. 10. Kidney cells of rat stained with trypan blue and vitamin C reagent. Black granules can be seen surrounded by a grey envelope, which in the actual preparations is stained blue by trypan blue. From Hirsch (1939), after Tonutti.

FIG. 11. Vitamin C preparation of human decidua cell, showing localization of vitamin C in the Golgi region. From Giroud (1938), after Tonutti and Plate.

PLATE 3

FIG. 1: *a, b, c, d, e, f, g*. Golgi apparatus in adrenal cortical cells of marsupials. *h*. Golgi apparatus in medullary cell of opossum adrenal. (Re-touched.) From Bourne (1934). *a* and *b*, $\times 2,500$; *c, d, e, f, g*, $\times 1,200$; *h*, $\times 1,800$.

FIGS. 2 and 3. Vitamin C preparations of cortical cells of rat adrenal. (Re-touched.) $\times 2,500$.

FIG. 4. Vitamin C preparation of medullary cell of rat adrenal. (Re-touched.) $\times 2,900$.

FIGS. 5 and 6. Medullary cells from ultracentrifuged rat adrenal stained to demonstrate the distribution of vitamin C. Most of the granules can be seen to occupy the position usually taken up by the Golgi apparatus in centrifuged adrenal medullary cells. In Fig. 5 some vitamin C granules are in the same region as the mitochondria. The chromatin of the nuclei is centrifuged to one end of the nucleus. (Re-touched.) Fig. 5, $\times 2,500$; Fig. 6, $\times 1,200$.

FIGS. 7 and 8. Cortical cells from the ultracentrifuged rat adrenal, stained to demonstrate vitamin C. The vitamin granules are in the position usually taken up by the Golgi apparatus in ultracentrifuged cortical cells. (Re-touched.) $\times 1,800$.

PLATE 4

FIG. 1. Intestinal cells of *Ascaris*. First two cells 20 minutes after feeding with iron sugar, second two cells 30 minutes after feeding with iron sugar. The first and third cells are stained by an osmium technique to demonstrate the Golgi apparatus. The correspondence between these two preparations shows that the Golgi apparatus plays an important part in the absorption of iron. From Hirsch (1939).

FIG. 2. Golgi apparatus in ameloblasts. As the cells commence to produce enamel the Golgi apparatus may be seen to reverse its position in the cell. From Hirsch (1939), after Beams and King.

FIG. 3. Regression of the Golgi apparatus in a neurone after section of its axone. From Hirsch (1939), after Cajal.

FIG. 4. Association of Golgi apparatus with secretion droplets in the submaxillary gland of the cat. n = nucleus. From Bowen (1926).

FIGS. 5 and 6. Showing hypertrophy of Golgi apparatus in neurones in fowls suffering from beri-beri. Fig. 5, normal cell; Fig. 6, cell from fowl suffering from beri-beri. (Re-touched.) After Chang (1932).

FIG. 7. Association of Golgi apparatus with secretory droplets in parotid gland of cat. n = nucleus. From Bowen (1926).

PLATE 5

FIG. 1. Typical morphology of the Golgi apparatus in the neurone of a healthy normal albino rat. Note the thinness of the anastomosing strands of the apparatus.

FIG. 2. Appearance of the Golgi apparatus in the neurone of a white rat killed 3 hours after a massive injection of morphine.

FIG. 3. Polymorphic structure of the Golgi apparatus in the neurone of a white rat killed 6 hours after a massive injection of morphine. The reticulum has completely disintegrated, giving rise to incomplete ring-like structures.

FIG. 4. Neurone of rat at same stage as in Fig. 3. Ring-like formations of the Golgi apparatus also present.

The photomicrographs for this plate were kindly supplied by Dr. E. S. Horning.

PLATE 6

FIG. 1. Neurone of white rat killed 7 hours after a massive injection of morphine. The Golgi apparatus is hypertrophied and fragmented.

FIG. 2. Neurone of white rat killed 8 hours after a massive injection of morphine. The Golgi reticulum is disintegrated through the body of the cell.

FIGS. 3 and 4. Neurone of white rat after massive injection of morphine, showing morphological variations of the Golgi apparatus.

The photomicrographs for this plate were kindly supplied by Dr. E. S. Horning.

CHAPTER VII

MICRO-INCINERATION AND THE INORGANIC CONSTITUENTS OF CELLS

By E. S. HORNING

i. INTRODUCTION

IN 1833 the French botanist Francois-Vincent Raspail⁷⁷ demonstrated the mineral structure of plant cells by subjecting sections of plant epidermis to a process of incineration in order to eliminate the organic matter. Since then many spasmodic attempts have been made by subsequent investigators to employ this method as a cytological procedure for determining the inorganic constituents of cells.

No significant advances were made, however, until Liesegang⁴⁷ in 1910, by selecting less primitive apparatus than previous workers, described in incinerated microscopical sections the topographical distribution of heat-resistant mineral ash of blood corpuscles in the capillaries of embryonic fish brains. He likewise described the ashed remains of such delicate structures as the retina in the developing eye, optic nerves, and individual neurones. Then followed the work of Herrera³⁰ in 1914, who applied micro-incineration to a study of various zoological and botanical problems. A few years later studies with this technique were extended by Naumann⁵⁷, Prenant⁷⁶, and Molisch⁵⁵. Although these workers stressed the possibilities of incineration as a microscopical method, their investigations met with only moderate success until another Frenchman, A. Policard⁶², in 1923 made considerable improvements in the technique. Policard introduced a suitable fixing solution and an electric quartz furnace by which the incineration process could be more adequately controlled. Scott⁹¹ subsequently added several modifications to Policard's technique, amongst which was the examination of incinerated preparations by dark field instead of oblique illumination. These modifications established a technique by which the inorganic residues of microscopical sections could be compared with stained control paraffin sections to a remarkable degree of accuracy. The failure of previous workers had been due to their inability to find a suitable fixing fluid and devise a controlled method of incineration.

When these preliminary technical difficulties had been overcome, many workers were at once attracted by this novel microscopical technique, and it was enthusiastically applied to an examination of material covering a wide biological field. Formerly the only available method

of determining the inorganic contents of cells was by a chemical analysis of tissues in bulk—a technique hardly adaptable for bacteria, inclusion bodies, protozoa, and other individual cell types. Papers describing the mineral components of inclusion bodies associated with certain virus diseases, bacteria, and protozoa soon appeared, while pathologists likewise applied micro-incineration to an extensive study of normal and diseased tissues.

One advantage of incineration is that it permits a microscopic examination of the relative topographical distribution of mineral ash within individual cells, giving an indication of its probable distribution in the living tissues and organs of the body⁹⁴. Certain technical difficulties occur, however, owing to the exceedingly small concentrations of the inorganic residues involved in thin sections suitable for microscopical examination. The main problem confronting investigators has been to discover analytical means by which quantitative and qualitative estimations of the inorganic constituents of incinerated sections could be determined.

From 1928 onwards the optical appearance of certain heat-resistant minerals in incinerated preparations examined in dark field, polarized and ultraviolet light, was recorded. This method, frequently used in conjunction with microchemical tests, was in some respects unsatisfactory and uncritical. It became obvious from the numerous papers published at that time that the interpretation of the visual appearances of the minerals forming the ashed residues was hardly sufficient to establish their chemical identities. The possibility of complex combination of the inorganic salts occurring during the incineration of the tissues was apparently not fully appreciated. Such limitations of micro-incineration as a histochemical technique were stressed by Scott⁹⁵ in a critical review published in 1933 and later in 1943. Nevertheless, even without accurate chemical identification of ash residues, many interesting observations resulting from the use of micro-incineration have been recorded, which will form the subject of a later discussion.

It was realized that if quantitative and qualitative means of identifying the inorganic constituents of cells and tissues could be discovered, an entirely new approach to cell physiology could be made. Researches in this direction were undertaken with the aid of the electron microscope, spectrographic analysis, and a photo-electric process. So far the most promising results were obtained in 1939 by the successful and ingenious application of a modification of the electron microscope, by which Scott and Packer¹⁰³ have succeeded in localizing mineral elements *in situ* in ashed tissue sections.

A short account of the technique of micro-incineration, its application to biological and pathological problems, and a survey of the results obtained will now be undertaken.

ii. TECHNIQUE OF MICRO-INCINERATION

a. Methods of Fixation

The most universal and convenient method of fixing fresh tissues for micro-incineration is that originally described by Scott⁹², in which small pieces of tissues are fixed for 24 hours in a solution containing 9 volumes of absolute alcohol to 1 volume of neutral commercial formalin. In order to ensure complete dehydration and to remove the formalin, all material is then passed through several changes of absolute alcohol, prior to clearing in xylol and embedding in paraffin. Absolute alcohol was selected as a fixative, since very few of the inorganic constituents common to protoplasm are soluble to any great extent in this fluid. Owing to the fact that absolute alcohol is such a poor histological fixative, and in order to minimize cell distortion, formalin was added to the alcohol. Scott⁹² found that analysis of the formol-alcohol solution, subsequent to its use for the routine fixation of tissues, failed to give any positive reactions for K, Na, Mg, or Ca. It may be assumed, therefore, that the fixation process does not extract appreciable amounts of inorganic substances before incineration, although it is possible that during the penetration of the fixative some shift in the localization of individual inorganic substances may occur in the cells and tissues.

According to Alexander and Myerson² tissues that have been preserved for several weeks, either in 10 per cent. formalin alone or in 95 per cent. alcohol, can be used for incineration, but they report that less brilliant ash preparations were obtained. Distortion owing to tissue shrinkage was commonly found. Gage²² advised fixation in a 3 per cent. aqueous solution of potassium bichromate with the addition of 10 per cent. neutral formalin, and suggests even the use of Helly's fluid. Washing the material in running tap-water and repeated immersion in 70 per cent. alcohol is required to remove the chromium. Experience has shown that fixation in either of the above solutions fails to give results as good as those obtained by the use of alcohol-formalin. For obvious reasons it is advantageous to avoid aqueous fixing fluids containing either chromium or mercury when preparing tissues for micro-incineration.

Alexander and Myerson² recommend the use of frozen sections of fresh material no thicker than 10μ , provided the sections are passed through graded alcohols and ether prior to incineration. This technique is also employed by Roddy⁸⁴. Tissues containing soluble or diffusible salts are sectioned by the freezing microtome and placed in xylol, prior to mounting on Corex D slides which have previously been filmed with glycerine-gelatine. Material, on the other hand, which contains soluble or diffusible salts is first fixed in 10 per cent. formalin prior to sectioning and mounting in ethyl alcohol. Although this method is time-saving, and can be carried out in under 1 hour, it is difficult to control the thickness

of frozen sections, and the subsequent loss or distortion of the soluble inorganic salts by treatment with low-grade alcohols must result in loss of detail after ashing. This method, however, is convenient for a rough quantitative estimation of ash distribution in pathological preparations.

The Altmann-Gersh freezing-drying technique undoubtedly has proved the most successful method for the preparation of thin sections of animal tissues suitable for micro-incineration. In fact by this process, which has been modified by Gersh²⁴, Bensley and Gersh⁷, and others, it has been possible to make most important contributions to the study of cytology as a whole. Among its many advantages are the instantaneous method of fixation and the sudden cessation of all metabolic activities and shifting of substances within the cells and tissues immediately before the fixation process is carried out³². The technique permits, moreover, the preservation of mitochondria and other cytoplasmic inclusions, and also of vitally stained preparations which are so often difficult to preserve in routine fixing solutions. The absence of cell shrinkage and no appreciable diffusion or displacement of the cell constituents make the technique very desirable for combination with micro-incineration.

In 1932 Gersh²⁴ developed the Altmann method of freezing animal tissues with liquid air and of subsequently drying them *in vacuo* at low temperatures. In the dried state such material is suitable for cutting hand-sections or for direct infiltration with paraffin so that the sections are prepared from material which has not come into contact with any of the reagents and solvents commonly used in microscopical techniques. An experimental analysis of the Altmann technique of freezing-drying has been published more recently by Simpson¹⁰⁸. Scott⁹⁵ was one of the first to apply this method in conjunction with micro-incineration. He found, however, that while tissues treated by the Altmann-Gersh technique produced an ash residue showing cytological structure, the results were not comparable with those obtained by the routine alcohol-formalin fixation. While at the University of Chicago the author had the opportunity of making a similar comparison, and confirmed Scott's statement. Scott also found that the distribution of inorganic salts, as seen in incinerated tissue sections, varied according to the temperature of dehydration. At that time Gersh²⁴ recommended that for histological purposes tissues should be dehydrated at -20°C . Scott pointed out that the temperature selected by Gersh as suitable for carrying out dehydration of the frozen tissues was above the eutectic point (-21°C .) of such universally distributed salts as sodium chloride. It was also found that freezing by liquid air produces in tissue cells a solid mass of ice crystals in equilibrium. Disturbance of this equilibrium by warming the frozen tissues above eutectic point would lead to diffusion and shifting of ions during the period of dehydration. Scott⁹⁸ then showed that

this objection might be overcome by dehydrating tissues at temperatures ranging from -38°C . to -78°C . The incineration of sections from this material yielded results closely comparable with those obtained by alcohol-formalin fixation. Further experiments led Scott⁹⁵ to recommend the use of alcohol chilled to -177°C . instead of liquid air. More recently Hoerr³² has confirmed the fact that the rapidity with which tissues are frozen determines the quality of the cytological fixation. He has stated that 'if the tissue is not frozen at the optimum rate, so much of the free water in the tissue may freeze out that the tissue is blown up by large ice crystals and after drying presents an extremely vacuolated appearance'. Hoerr recommended more rapid freezing of tissues by means of pentane chilled to -131°C ., or iso-pentane chilled to -195°C ., followed by dehydration at a temperature of -30°C . He criticized Scott's use of chilled alcohol on the ground that alcohol at -177°C . is so viscous that the freezing is not as rapid as that obtained by the use of pentane. In addition, it was shown to be advantageous to allow tissues to warm up very slowly after the initial freezing to the temperature of dehydration.

Scott and Packer¹⁰³ expressed the opinion that, provided material is previously prepared by the pentane modification of the freezing-dehydration method, there exists little chance of 'any perceptible shift in the cellular location of the inorganic constituents'. Their opinion was based upon tissues examined by the electron microscope after incineration. More recently Scott⁹⁸ has returned to the use of liquid air and, provided the material is dehydrated *in vacuo* at -63°C . and subsequently infiltrated with water-free paraffin, it was concluded that no appreciable shift occurred in the inorganic salts, the addition of water from external sources having been eliminated.

Attempts to apply the freezing-drying technique to plant tissues did not give satisfactory cytological results at first. The majority of plant cells possess higher mineral salt concentrations and their water content is higher than in animal cells. Goodspeed and Uber²⁶ introduced new modifications of the technique to overcome these difficulties, and they recommended particularly a slower rate of dehydration for plant tissues.

b. *Micro-incineration*

In order to obtain the most satisfactory cellular details in ash residues, sections should be no thicker than 5μ . Series of sections should be cut in which alternate ones are kept for incineration, the remainder being mounted and stained in the routine manner for controls. Only the best-quality glass slides are suitable for mounting sections for incineration. The sections should be smoothed out by using either absolute alcohol or liquid petrolatum. Care must be taken to avoid contact with water, which might involve a loss or disarrangement of

the soluble inorganic salts. The contamination of sections by dust must be avoided.

The glass slides, on which the sections are mounted, are placed on small quartz plates (see Plate 1, Fig. 1), which lie horizontally in the furnace. One type of electric quartz furnace recommended is illustrated on Plate 1. A series of calibrations on the rheostat are used to mark a range of temperatures suitable for different types of material. Although Tschopp¹¹¹ advises passing oxygen through the furnace during the incineration, and Schultz-Brauns⁸⁸ employs nitrogen, the air currents set up by the heating of the quartz tube appear to be sufficient to provide ample oxygen for incineration. The use of gases enables incineration to take place at lower temperatures, whereby the greater proportion of chlorides in the ash may be retained. Policard⁶⁶ for this reason has used a mixture of nitrogen together with a small concentration of oxygen in order to bring about a more rapid oxidation. Provided the preparations are not incinerated too rapidly, satisfactory results are nearly always obtained by burning in air. The temperatures required to burn off organic matter vary with different tissues. Thus, certain types of neoplastic growths (tar tumours in particular) and mammary glands fixed during functional activity remain carbonized longer than the majority of normal tissues. Protozoa and tissue cultures incinerate very rapidly. Generally speaking, the incineration of normal tissues should extend over periods ranging from 45 to 55 minutes, during which the temperature should be increased by approximately 70° C. every 5 minutes to reach a maximum temperature of 650° C. Tissues containing high concentration of collagen or elastic fibres tend to shrink when the temperature is first raised to 70° C., but this can be avoided if the rise in temperature is spread over a longer period. Similarly, thin sections of brain require a more gradual rise in temperature during incineration, to avoid distortion. Scheid⁸⁶ has found that thick brain sections incinerate more successfully if the phospholipids are first extracted with suitable solvents.

Scott¹⁰⁰ has described a type of micro-incinerator which is a modification of the one already described, and which appears to have several advantages. His furnace consists of a 24-inch quartz tube with differentially heated segments. The sections for incineration are carried through the quartz tube on a slowly moving automatic trough. The first segment is heated to 80° C. to drive off water and the most volatile constituents of the tissues, the second is heated to 300° C., and the third segment to 600° C. The slides pass through the 24-inch tube in about 35 minutes. The advantages are that several slides can be incinerated at the same time, and that all slides receive the same heat treatment.

When the incineration is completed, the slides are allowed to cool slowly before removal from the furnace. A cover-slip is then placed

over the ashed preparations, the edges of which are sealed with warm paraffin, thus permitting the use of an oil-immersion objective if required. The sealing also prevents the absorption of moisture and efflorescence of the ash. The incinerated sections should be examined at once by direct illumination to ensure that complete incineration has been achieved. Carbon appears black or brownish by transmitted light, and consequently areas in a section which have not been completely incinerated can be easily detected. The ash distribution of the incinerated section is then viewed by dark field illumination. With this method the contours and relative densities of the ash are revealed with remarkable brilliance. A dark field condenser, allowing a rapid change from dark field to direct illumination, will be found useful for the examination of the incinerated sections and the stained control preparations. High-power investigation of ashed sections can be undertaken with an apochromatic homogeneous oil-immersion objective, provided an iris diaphragm is fitted within it.

Two kinds of microscope-incinerators have been described by Uber and Goodspeed¹¹², and Godlewski²⁵, which permit continuous observation of the sections during the process of incineration. The former authors employed their apparatus for recording observations of the behaviour of sections of plant tissues during the burning process. This instrument consisted of a 25×50 mm. aluminium slide chamber, wound with resistance wire, in which temperatures reaching a maximum of 650° C. were attained. Temperatures were read by means of a chromel-alumel thermocouple. A simple doublet lens formed a window which constituted the microscope, giving a magnification of $\times 200$, according to the ocular employed. By this ingenious method they were able to determine at which temperature carbonization and the maximum shrinkage of plant-tissue sections occurred.

The apparatus described by Godlewski²⁵ allows continuous observation of a section by transmitted light during the incineration process, at a magnification of about 250 diameters. A small incinerator attached to the microscope stage is used for animal tissue sections in combination with two glass cooling-chambers. One of these is made to insulate the incinerator from the microscope stage, and the other surrounds the objective, moving with it so that the lens can be lowered to within its normal working distance despite the high temperature of the section. Mounted on a mica sheet, and held in a frame, the section is inserted into the incineration chamber so that it lies just above a mica strip, on which is wound a wire-heating element of Kanthal A alloy. The cooling-chamber encasing the objective is made of glass except for a small mica window sealed into its base. Water is circulated through the chamber so that the lens and its mount are kept cool, as well as the mica window interposed between the lens and the section. The light necessary for

microscopical observation passes from the substage condenser, through the heating element, and so through the section, the mica window, and the water, to the objective.

With this apparatus it is possible to view the section at all stages of incineration while the temperature is rising and so to detect changes such as melting, fusion, cracking, or gross movements of the substance of a section before it carbonizes and is ultimately reduced to an inorganic residue.

Direct observations of sections during incineration by the methods just described do not suggest that any serious shifting in mineral salts occurs during the burning process. This evidence is supported by Scott⁹⁹, who states that motion-pictures of incinerating sections of skeletal muscle and of anterior horn cells taken at a magnification of 700 to 800 failed to reveal, when projected on a screen, any mechanical shifts of the inorganic constituents during incineration.

Policard⁶⁷, however, is of the opinion that the ultramicroscopic structure of the substances in a paraffin section must influence the form the ash finally takes on completion of the incineration process; whether, for instance, coarse or fine granules, or diffuse deposits, are left in such areas as the nucleus or cytoplasm. Moreover, during heating and charring it is clear from the examination of relatively simple structures like red blood corpuscles that the way in which the material adheres to the glass slide is also very important, because retraction of fragments during heating will partly determine the form of the final ash. The ideal method of incineration would be to have the section so securely stuck to the slide that the initial heating could cause little or no movement of the organic materials up to the end-point of incineration.

iii. THE IDENTIFICATION OF HEAT-RESISTANT MINERALS IN MICRO-INCINERATED SECTIONS

The precise chemical identification of inorganic substances *in situ*, within individual incinerated cells, has proved the most difficult aspect of the technique. Unfortunately, the inorganic ions in cells often form complex combinations with organic substances, and doubtless certain complex chemical reorientations must occur during the process of incineration. Moreover, it has been calculated that calcium, for instance, is present in the order of 1×10^{-13} grammes per cell of 1,000 cubic μ^{95} . Such a minute concentration falls outside the limits of many histochemical tests. Recent analytical methods, which are still in an experimental phase, however, have begun to yield promising results.

Qualitatively, there are two methods for the identification of inorganic substances in ashed material. The first, which has been mentioned already, deals with the visual appearances of the ash residues as seen by direct and dark field illumination. The second involves the application of the electron microscope, which has recently been modified for

the special purpose of examining incinerated preparations. The latter method obviously provides a more critical means of analysis.

a. Optical Differentiation

The optical method of identifying the inorganic constituents of incinerated sections, according to their visual appearances when observed by dark field, or polarized or ultraviolet light, should be used with caution. Of those substances present in the ash in the form of oxides only calcium, iron, and silica can be recognized with any degree of certainty. Calcium and magnesium compounds give a white, amorphous ash, and there are no means of differentiating one from the other by their optical appearances in dark field. The presence of calcium oxide can be detected by the gypsum reaction previously described by Moreau⁵⁶. Silica is present as a silico-calcareous compound. It can be recognized by its crystalline appearance and by double refraction when viewed by polarized light. The colour of iron oxide is sufficiently intense in some incinerated sections to give the ash in which it is mixed a yellow or reddish tinge. Deep red coloration, according to Policard⁶³, indicates the presence of free iron. Before the presence of iron can be assumed with any degree of accuracy it is essential first to examine the preparation by transmitted light, since any carbon residues may yield colour values similar to those of ferric oxide when viewed in dark field illumination.

Policard and Okkels⁷¹ have claimed to detect the presence of uranium salts in incinerated sections from animals previously poisoned by uranium, by means of their fluorescence in ultraviolet light. Since extremely small amounts of impurities other than uranium often give a fluorescence, the identification of uranium by visual appearances is open to error. Policard and Pillet⁷³ have attempted to identify the chlorides of sodium and potassium in tissue sections by converting these compounds into sulphates before incineration. They exposed sections to the action of sulphuric anhydride, with the result that the sulphates formed in the tissues could be identified in the ash after micro-incineration. In the case of lead introduced into tissues and organs of experimental animals, Okkels⁵⁸ used hydrogen sulphide to form lead sulphide from the ash residues. The black particles of lead sulphide are visible by transmitted light, but it should be noted that the identification of this substance can easily be confused with carbon deposits remaining in the preparation as the result of incomplete incineration.

b. The Electron Microscope

Busch^{8,9} first described the principles of the electron microscope, which was subsequently modified for use in biological research. The contributions of the electron microscope to medical research have been

reviewed recently by Zworykin, Morton, Ramberg, Hillier, and Vance¹¹⁸. The most important technical improvements were achieved by Ruska⁸⁵ in 1933, the power of magnification being increased to 12,000 diameters by the use of two magnetic 'lenses' (iron-encased magnetic spools). In 1935 Martin, Parnum, and Speak⁵³ modified the electron microscope for biological research by constructing an instrument which permitted the object to be viewed simultaneously with an ordinary microscope. The magnifications obtained by this instrument were somewhat lower than those obtained by Ruska. It is now possible, with the more recent models, to examine biological material at a magnification of 35,000 diameters¹¹⁸ on the fluorescent screen of the microscope, and up to 100,000 times by optical enlargements.

McMillen and Scott⁵² in 1937 succeeded in designing an electron microscope capable of being adapted for the identification of certain mineral elements present in ashed tissue sections. The lens of their instrument was a short iron-encased spool similar to that described by Kroll⁴³. A further modification was described by Scott and Packer¹⁰⁴ two years later.

When metals or metallic compounds are heated *in vacuo*, streams of electrons are emitted. The electron microscope makes use of such a beam of electrons in an evacuated tube instead of light rays, as in the case of the optical microscope. By employing suitable magnetic fields placed at different points along the length of the evacuated tube, it is possible to focus the electron beam in such a way that an image is produced on a fluorescent screen at the end of the tube. The magnetic fields act as 'lenses' and, in the same way that the focal length of an optical system depends upon the wave-length of light, so does the focal length of a magnetic lens vary with the wave-length of the electrons. In order to obtain a magnified image of a tissue section by the electron microscope, the electron beam can be made to originate from the minerals of the actual section itself, or electrons from another source can be directed through a tissue section before coming under the influence of the magnetic lenses, to fall finally on the fluorescent screen. The number of electrons emitted by minerals under these conditions varies with the temperature and the type of compound. Hence it is possible to distinguish inorganic substances by their differential emission of thermally excited electrons.

Scott and Packer¹⁰³ in preliminary tests showed that they could differentiate between the different emissions produced in a complex mixture of metallic salts, such as exists in ashed sections. Minute amounts of metallic salts which were placed on the cathode could be readily identified on the fluorescent screen. Numerous similar experiments were repeated under a variety of conditions, after which it was found that all salts present in the test samples, with the exception of

iron, produced a recognizable emission. The most brilliant images on the fluorescent screen were obtained from calcium and magnesium. Tests were likewise carried out which more nearly approached the conditions existing in tissue sections. A solution of ashless gelatine impregnated in separate areas by saturated solutions containing calcium, magnesium, iron, sodium, and potassium chlorides was hardened in 10 per cent. formalin prior to dehydration in alcohol and embedding in paraffin. Sections of 10μ were cut and immediately placed on a coated cathode. After heating slowly for a period of 90 minutes the cathode was raised to a temperature sufficiently high for the emission of electrons. With the exception of iron all the chlorides could be identified on the fluorescent screen, the brightest images again being obtained in the case of calcium and magnesium.

Before proceeding to identify mineral substances in tissues by the electron microscope, Scott and Packer¹⁰³ treated all material by the modified Altmann-Gersh frozen-dehydration technique. Sections from the frozen-dried material were cut at 10μ and placed on a nickel cathode, which had first been coated with a mixture of 40 per cent. barium and 60 per cent. strontium carbonates carried in a 2 per cent. solution of nitrocellulose in amyl acetate. The cathode coating was allowed to dry before the tissue sections were attached. This coating brings about an 'activation' phenomenon whereby sufficient electrons are emitted to give adequate contrast in the image. After attaching the section by flattening it on the surface of the coated cathode, the vacuum pumps were set in motion and, when a suitable vacuum had been obtained, the filament current was switched on to heat up the cathode and bring about a gradual incineration of the section. The heating period lasted for about an hour⁵². Following successful incineration, the high voltage and lens currents were then switched on and the electron image of the object was produced on the fluorescent screen.

By this ingenious technique Scott and Packer¹⁰³ have so far been able to obtain emission pictures showing remarkable structural and cellular detail, due to the presence of magnesium and calcium in the ashed preparations. These authors state that by a further modification of the instrument they should be able to localize sodium and potassium, which at present are both lost from the tissue section during the initial heating and before the electron image is obtained. When further technical modifications of this instrument permit the identification of other mineral elements, a great advance in histochemistry will have been achieved. In fact, the successful adaptation of the electron microscope to a mineral study of biological tissues opens up an almost unlimited field for future cytological research. The relative qualitative changes of the inorganic constituents during cellular differentiation in the developing organism, some details of which have already been indicated

by ordinary micro-incineration methods, are lines of investigation to which this new technique might be applied with important results. Likewise, the study of the changes in mineral substances associated with various diseases opens up a new field for the experimental pathologist. The determination of qualitative mineral changes *in situ*, within individual cells, would give the cytologist an insight into the role which the inorganic constituents play in health and disease.

c. Spectrographic Analysis

Histo-spectrography, by means of which small areas of tissues are subjected to spectrographic analysis, has been developed by Policard and Morel⁷⁰, Gerlach and Gerlach²³, Benoit⁶, and others. This technique has been used in conjunction with micro-incineration for ascertaining the mineral elements present in microscopic areas of incinerated tissues. By passing a high-frequency spark through a selected area of tissue, and analysing the rays emitted with a quartz spectrograph, a spectrogram is obtained which reveals lines characteristic of the mineral elements volatilized by the spark as it passes through a selected area of the tissue section. Scott and Williams¹⁰⁷, using a Gaertner 1,250-watt quartz spectrograph, have obtained strong lines on their spectrogram of Ca, Mg, K, Na, Cu, and P, in a variety of tissues examined.

There are certain technical difficulties in the use of this method as a means of analysing ash residues. In the first place, it cannot be employed for detecting the intracellular distribution of minerals. The burning produced by the high-frequency spark extends over an area of tissue consisting of many hundreds of cells. Unfortunately, as Scott⁹⁵ has shown, areas of tissue smaller than 1 mm. in diameter do not contain sufficient salts to register a recordable emission spectrogram. Furthermore, if this method is to be employed with micro-incineration, the selection of electrodes must be undertaken with caution, since certain elements of the electrodes are likely to appear in the spectrum. Scott and Williams¹⁰⁶ have eliminated these difficulties, and they have simplified the technique of spark spectrography by screening the electrodes and arranging that the material to be analysed is burnt in the centre of the spark. Yogada¹¹⁶ has described a modified technique adapted for examining plant material. Spectrographic analysis at present is useful in a limited way for the estimation of elements in ashed material, but since the results obtained cannot be referred to cellular structures, the method achieves little more than the cruder methods of the analysis of tissues in bulk.

d. Photo-electric Method

The accurate estimation of the amounts of mineral residues in incinerated cells provides yet another problem of the technique of micro-

incineration. A rough idea of the comparative amounts of ash in cells and tissues can be obtained by the visual inspection of the relative densities of the ash in dark field illumination. A finer visual analysis is impossible. In the first place, the quantitative determination of the ash is dependent on the relative thickness of the incinerated sections. Variations in thickness are difficult to avoid even with the best microtomes.

Schultz-Brauns⁸⁹ has devised a technique for the quantitative photography of incinerated sections based upon the standardized development of exposed photographic plates. This method has met with little success. Scott⁹⁶ has attempted to measure the intensity of light reflected from the mineral particles in ashed preparations, assuming that the reflected light was proportional to the amounts of ash in different areas of an incinerated section. Preliminary experiments with colloidal solutions indicated that, with a constant source of illumination, the intensity of light reflected from particles in the preparation was proportional to their number. Similar experiments with incinerated material showed that the same principles were involved. With the aid of a specially designed photomicrographic apparatus, a small area of a microscopic field under dark ground illumination is selected and focused by a plano-convex lens on the sensitive plate of a photo-electric cell. The current generated, when the light from the microscope strikes the photo-electric cell, is passed through an amplifier and its output measured by a galvanometer. The amount of light reflected in dark field is so small that the current generated in the photo-electric cell has to be amplified 40,000 times before a reading on a sensitive galvanometer can be recorded. Although this method does not give an analysis in terms of the weight of ash from individual cells, it does permit the mineral residues of a selected group of cells to be compared in mass with those of another group or layer. These quantitative variations of the inorganic residues in normal and pathological cells of the same type are sometimes important to the pathologist. The investigation of pathological calcifications and other allied phenomena by a quantitative micro-incineration method has given some very interesting data, which will be discussed in a later section of this review.

Finally, the important question arises as to whether the distribution of ash obtained in incinerated sections is comparable to that existing in the living tissues and cells. The possibility of a shift of the inorganic salts occurring during fixation and incineration must be considered. In this connexion Scott⁹⁴ has demonstrated that photomicrographs of living cells taken with ultraviolet light of a wave-length of 2,750 Å show an absorption of the rays in precisely those areas of a section which yield ash residues after incineration. One is impressed, moreover, with the remarkably delicate orientation of ash residues resulting from the

incineration of secretory granules within the cytoplasm of glandular cells, of chromosomes during cell division, and of structures like cilia. It has been noted, for instance in adipose tissue, that the spaces occupied by fats are devoid of ash in incinerated sections, whereas the cell membrane surrounding these spaces yields an ash which corresponds exactly in position and thickness to the membrane observed in stained control preparations. From these and other observations it is safe to assume that the elimination of the organic substances by micro-incineration leaves the inorganic residues in much the same topographical position within a section as exists in the living tissues.

iv. NORMAL CYTOLOGY

a. Blood

The precision of the micro-incineration technique is fully demonstrated when incinerated films or sections of blood or protozoa are examined by dark field illumination (see Plate 2, Figs. 1 and 2). The mineral residue of a red blood corpuscle appears to consist of a combination of potassium and sodium with enough iron oxide to give the ash a slightly yellow colour. The more complex white corpuscles of the blood, such as lymphocytes and eosinophil leucocytes, leave a more organized ash. They can be readily identified by their inorganic remains. The polymorphonuclear leucocytes, as Scott⁹⁷ has demonstrated, possess cell membranes which are clearly defined by an orientation of finely divided ash. There are mineral-free areas in their cytoplasm. Scott has even been able to detect the mineral residues of burnt blood-platelets which are indentified without difficulty, since the blood-plasma leaves no detectable ash.

b. Cell Division

The behaviour of the inorganic salts during mitosis in tissue cells is an interesting phenomenon which was first described by Scott⁹¹. The ash from the resting nucleus shows less iron than one would expect and, according to Scott, the bulk of the nuclear ash consists of slightly water-soluble salts which are probably calcium and magnesium. The nucleolus in the resting cell is inorganically differentiated from the remainder of the nuclear chromatin. Most nucleoli contain appreciable amounts of birefringent ash with iron oxide. Both the nuclear and cell membranes in healthy normal cells show a delicate orientation of mineral ash. The cytoplasmic ash in most cells, with the exception of neurones, is thinly and evenly dispersed. Neither the mitochondria nor the Golgi apparatus can be distinguished within the general cytoplasmic ash. At the commencement of mitosis, the inorganic salts of the whole cell appear to become concentrated in the chromatin prior to chromosome formation. This phenomenon is most noticeable in the late prophase, and the fully

formed chromosomes of the metaphase consist of aggregations of almost solid white ash. These details are best illustrated during embryonic development in the chick.

In the majority of cells there is a marked tendency towards a concentration of mineral substances along free surfaces, as, for example, in the free borders of the stomach epithelial cells and also in urinary epithelium. This phenomenon^{103,104} is even more marked in the case of the striated border of intestinal epithelial cells previously fixed by the alcohol-formalin method. Such an accumulation of inorganic salts has been reported in the free surface of absorptive epithelial cells by Macklin and Macklin⁵¹ and by Scott⁹⁷. Later, Scott and Packer¹⁰³ confirmed these findings and analysed the salts in these regions by the application of the electron microscope. The ash concentrations were found to consist mainly of magnesium and calcium.

Space will not permit a discussion of the general distribution of mineral substances in normal tissues, nor is there any need, as Scott⁹⁷ has most ably dealt with these problems in a previous communication.

Before passing on, however, a brief reference will be made to several interesting cytological observations recorded in this field. It was always problematical as to whether the pigment in the Ciaccio cells of the mammalian pineal body contained appreciable quantities of iron. Baginski⁴, from incinerated sections of the pineal, was able to determine that the Ciaccio cells contained appreciable quantities of iron. Funaoka and Ogata²¹ recorded some interesting observations on the nuclear ash content in the cells of the ovarian tube in *Ascaris megalocephala* and also in the stamens of *Vicia faba*. Kooyman⁴² has studied in great detail the mineral distribution in the cells of the skin. Also, Policard, Noel, and Pillet⁷⁴ described changes in the mineral structure of organs produced by experimental metabolic changes.

c. Protozoa

Micro-incineration of various types of protozoa has added interesting data to our knowledge of these organisms. Differences between the mechanisms of nutrition in various species of infusoria appear to be related to the concentration and distribution of their ash residues. Policard⁶⁴ in 1929 was the first to examine the mineral 'skeleton' of a protozoan cell by micro-incineration. He selected for this purpose the vorticellid *Carchesium polypinum*, and described the ashed remains of the vegetative structures as distinct clumps of ash 3 to 4 μ in diameter. The ash of these bodies was whitish in appearance and almost crystalline in structure. The ash of the endosarc contained appreciable amounts of iron, while the myonemata and the basal granules yielded finely orientated white ash residues.

Scott and Horning¹⁰¹ described the ash residues of the binucleate

infusorian *Opalina*. They were able to elucidate the cytoplasmic nature of the so-called vegetative bodies, which had previously been the subject of much controversy (see Plate 2, Figs. 1 and 2). Several protozoologists^{31,41,109} had contended that these cytoplasmic structures, as previously identified by somewhat capricious cytological techniques, represented Golgi substance. Incinerated preparations of the organism showed that these vegetative bodies did not consist of Golgi material, since they left a distinctive and organized clump of calcium-containing ash. Bearing in mind the method of feeding of this astomatous ciliate and the theory that mitochondria are associated with enzyme activity, it was suggested by Richardson and Horning⁸³ that these associated vegetative granules are synthesized under the influence of the mitochondria. Later, Horning and Scott,⁴⁰ in a more detailed and comparative study of the differences in the ash organization between a saprozoic and a holozoic infusorian, were able to demonstrate that the mineral distribution in the two types of protozoa could be correlated with their different mechanisms of nutrition. The two types of infusorian selected were *Nyctotherus cordiformis* and binucleate Opalinids infecting *Rana pipiens*.

Since Opalinids are saprozoic and astomatous, the concentrations of inorganic material seen occasionally within the surface cytoplasm may possibly be associated with the mechanism of nutrition. The nature of the mineral salts aggregated in the surface cytoplasm varies considerably in Opalinids infecting different hosts. In some species this ash residue appears to contain large traces of iron and calcium, while in others there is very little ash in the peripheral areas. At times the concentration of inorganic salts in Opalinids entirely masks such structures as the myonemata and basal granules, which are usually visible in incinerated starving Opalinids. Detailed comparative examination of this region of the cell shows not only quantitative, but also qualitative, variations in the inorganic salts, suggesting that this phenomenon is in some manner correlated with the diffusion of digested or digestible material through the cuticle into the protoplasm of the organism. This is of interest in view of the findings of Macklin and Macklin⁵¹ and Scott and Packer¹⁰³, who showed that aggregations of inorganic salts occur in the free margins of absorbing intestinal epithelial cells.

When the ash residues of the protoplasm and its various inclusions in *Nyctotherus* and *Opalina* are compared, interesting differences are revealed between the general organization of mineral substances composing the skeleton of the holozoic and saprozoic forms. Whereas in *Opalina* the cytoplasm is relatively free from ash, except for the presence of the inorganic remains of the vegetative granules, the general cytoplasm of *Nyctotherus* yields a fine network of mineral residues. Another interesting characteristic of incinerated sections of *Nyctotherus* is the

vacuolated appearance of the cytoplasm. These vacuoles are clearly outlined by a deposit of concentrated ash, and they are similar to the vacuoles in the endoplasm of *Vorticella* described by Policard⁶⁴. The nature of the inorganic accumulations forming the vacuolar wall is of considerable interest and importance, since they are relatively rich in iron. The presence of iron at the periphery of these vacuoles might indicate an increased rate of oxidation in these areas. It has been suggested that phosphatides aggregate at the interface of the cytoplasm owing to their capacity to reduce surface tension according to the Gibbs-Thomson law⁵⁴, and possibly they do so in combination with inorganic substances like iron.

Unlike the nuclei in *Opalina*, which are characterized by little or no mineral residues, the macronucleus of *Nyctotherus cordiformis* contains so much mineral material that it crumbles when sectioned and cannot, therefore, be differentiated from the micronucleus in ash preparations. Other structures of these types of ciliates, readily revealed by micro-incineration, are the basal granules, the cilia, and myonemata. The clarity with which the ash of delicate structures such as these is shown by the technique, together with general differences in organization and orientation of the inorganic material, opens the way for further studies on these infusorians.

d. Multinucleated Giant Cells

Okkels⁵⁹ produced multinucleated giant cells experimentally in the peritoneum of the small intestines of mice and examined them in incinerated sections. He found that the ash of the Langhans giant cells was relatively insignificant. The central region of the cell did not contain more calcium than the peripheral cytoplasmic zone. This was a significant observation, being contrary to the general idea that necrotic changes are always taking place in the centre of the Langhans cells.

In cases of inflammatory processes occurring in various non-infective conditions, Okkels found that giant cells were also poor in ash residues. On the other hand, it was interesting to note that giant cells found in relation with human carcinoma of the breast always contained calcium ash. It was further observed that the macrophages containing blood-pigment in inflammatory areas were extremely rich in iron-containing ash.

e. Tissue Cultures

By means of a slight modification in technique, Horning and Scott³⁸ succeeded in incinerating tissue cultures. Tissues from 7-day chick embryos were selected for these experiments. Perhaps the most interesting observation recorded was that all cells in the cultures, especially

clasmotocytes, which generally contain traces of iron in tissue sections, showed no such traces when ashed in cultures. This was possibly due to the fact that the tissues had been cultivated *in vitro* in a medium entirely cut off from a blood-supply. 'Pure' cultures of epithelium and of fibroblasts were cultivated separately and compared after incineration. The heart fibroblasts contained considerably greater amounts of clumped calcium ash than the cells forming the sheet-like cultures of epithelium. In the latter the cytoplasmic ash was more evenly dispersed, except in the mobilized cells at the periphery of the culture medium.

f. Nerve Cells

Alexander¹ has examined the mineral contents of neurones in human material. The cytoplasm of ganglion cells in the cerebral cortex was found to be rich in mineral substances, which varied in distribution in different parts of the cell. The dendrites and the nucleoli left a distinctive ash, but the remainder of the nucleus was ash-free. The Nissl bodies were found to contain a concentration of calcium. In the general cytoplasm traces of iron and calcium could be identified. The neurofibrillae, as might be expected, were entirely free of mineral residues, and the axon hillock and axis cylinder contained very small amounts of ash, which did not appear to consist either of calcium, iron, or silica. The myelin sheaths left no residues at all. It was further noted that the ash of neurones examined during embryonic development was greater in concentration than after differentiation had been completed.

g. Smooth and Striated Muscle

Scott⁹⁸ has studied the mineral content of muscle by means of incineration as well as by the electron microscope. In smooth muscle the nuclear membranes and the myofibrils yield a distinctive ash, which shows a tendency to aggregate at the cell surfaces. The myofibrils retain their individuality in the ash up to the very edge of the fibres. 'Contracture waves' crossing the fibres were inorganically represented by marked concentrations of ash of a distinct bluish-white colour, which differs from that seen in the uncontracted portions of the fibres.

In striated muscle the ash forming the striations has been found to contain iron. Alternating with the striations were areas which were optically mineral-free. The more concentrated ash deposits corresponded with the anisotropic disks, and in all probability the ash-free areas indicated the position of the *J* or isotropic disks. In well incinerated preparations even the *Z* band of the *J* disk and that of the *Q* disk could be detected. In striated muscle the sarcolemma left a white ash containing calcium.

With the aid of the electron microscope, Scott and Packer¹⁰³ made

a study of the location of calcium and magnesium both in smooth and striated muscle. Strips of smooth muscle were taken from the wall of the duodenum of cats and frogs. The pieces of muscle were frozen in liquid air while actively contracting and then dehydrated *in vacuo* before infiltration with water-free paraffin, according to the method already described. This treatment of the muscle precluded any shift in mineral salts within the tissues. In no instance was either calcium or magnesium found to be located outside the cell membranes. The muscle-fibres from the frogs showed a marked concentration of salts within the sarcoplasm, and some indication of the protoplasmic bridges which can be demonstrated in routine stained sections to cross the intervals between adjacent fibres. Otherwise no calcium or magnesium salts could be demonstrated in the endomysium. Scott⁹⁸ has pointed out that these observations with the electron microscope are of interest in view of the recent work of Steinbach¹¹⁰, who contended that calcium is always present in cells in lower concentration than in the surrounding medium.

According to Carey and Zeit¹¹ smooth muscle from guinea-pig intestine or uterus, and the so-called transitional muscle of the bird's gizzard, develop contraction compression waves if the tissue is transferred from Locke's solution at 42° C. to the same medium at 0° C., and then fixed immediately afterwards. Similar serial contraction compression waves occur in frog skeletal muscle-fibres after sudden warming or cooling. Sections of the fixed muscle exhibit dark-staining nodal stripes (pressure nodes) and pale-staining tension internodes, which correspond in incinerated sections to heavy ash deposits alternating with internodal areas which are relatively ash-free. The regional distribution of ash in skeletal muscle is related to nodal crowding of narrow Q bands with internodes of coarse, widely spaced Q bands.

h. Mammary Gland

The effects of radium radiations upon the mammary epithelial cells of the mouse during di-oestrus, oestrus, pregnancy, and lactation were examined by Horning³⁵ with the micro-incineration technique. No specific variations were found in the distribution of the inorganic constituents of the unirradiated mammary glands fixed during di-oestrus and oestrus. During di-oestrus the sparse secretion often seen in the ducts in histological preparations was found to leave no ash after incineration. During early and late gestation differences between the amounts of ash in the alveolar epithelium were more marked. Taking into account the morphological fluctuations of the mammary gland cells during pregnancy, the variations in the cytoplasmic ash could be correlated with the degree of cellular differentiation and secretory activity.

White calcium-containing ash of the alveolar cells, in material

incinerated at 6 days' gestation, was very diffusely arranged, except in the nuclei and towards the apical cytoplasm, where it was deposited in thicker concentrations. Material on the 17th day of gestation gave ash residues indicating a greater concentration of inorganic salts both in the epithelium and in the distended lumina of the alveoli. The epithelial cytoplasm presented a honeycombed appearance owing to the complete incineration of the fat droplets, which left no inorganic residue. Dense accumulations of calcium were found around the periphery of the spaces once occupied by the fat secretion in the cytoplasm. The white calcium ash derived from the incinerated milk in the distended lumina was more diffusely deposited than in the epithelial cytoplasm. The micro-incineration technique demonstrated very clearly the fluctuations in the inorganic content of the milk secretion. The maximum ash content was reached from the 3rd to the 6th day of lactation, when the milk in the greatly distended alveoli yielded large concentrations of calcium residues (see Plate 3, Figs. 1 and 2). Material fixed on the 9th to the 11th day of lactation showed relatively less inorganic salts in the lumina. Owing to excessive concentration of ash within the secretory epithelium during early lactation, especially on the 3rd day, cellular details became more difficult to distinguish. The distended alveoli appeared as irregular rings of white ash, with the secretion appearing less homogeneous than that found during gestation, owing to the greater fat content.

Radium treatment did not produce any variations in ash distribution of the duct epithelium during di-oestrus and oestrus. Slight but variable increase in ash could be detected in the hypertrophied cells which had been irradiated on the 10th day of gestation and incinerated 5 days afterwards. More specific effects were obtained by irradiating the mammary gland on the 3rd day of lactation. Material fixed 3 days after irradiation showed a striking increase in the calcium content of the milk secretion as compared with unirradiated material at the same stage of lactation (see Plate 3, Fig. 2). There was no visible effect upon the ash content of the secretory epithelium. The distended alveoli contained a coarsely granular white ash which, owing to the fact that the fat secretion was inhibited by radium treatment, was more evenly distributed than in the control unirradiated sections of the gland (see Plate 3, Fig. 1). It is of interest to record that in the inorganic constituents of milk secretion a marked increase occurs following irradiation, whereas the fat secretion is inhibited.

i. The Formation of the Avian Egg-shell

In the so-called uterus at the terminal end of the oviduct of birds a remarkable mobilization and secretion of mineral salts occurs at repeated intervals to form the calcified shell of the egg. It has been calculated, in the case of the domestic hen, that approximately 5 grammes of nearly

pure calcium carbonate become deposited in the course of the 20 to 24 hours' interval during which the egg remains in the uterus. Sections of the uterine mucosa, taken at stages when no shell is being secreted and when a shell is in process of formation, show conspicuous differences in ash content (see Plate 4, Figs. 1 and 2). Richardson⁸² has demonstrated that the areas of uterine epithelium actually in contact with a developing egg-shell are much richer in mineral residues than adjacent portions of the same epithelium which are not in actual contact at the time of fixation. Unfortunately, the micro-incineration technique did not reveal the mechanism by which the calcium is deposited into the uterine lumen, despite the fact that secretory granules in the uterine epithelial cells were found to leave a well-defined ash residue. Since these studies were made, the modern improvements of freezing-drying fixation and the electron microscope have been combined with micro-incineration, and it should be emphasized that there is room for much further work with these techniques in an attempt to explain this extraordinary phenomenon of calcification.

j. Teeth

The mineral distribution in the developing teeth of man, dogs, cats, and rodents has been studied by Hamp²⁸. The findings in incinerated sections of the dental tissues were checked by spectroscopic analysis. The cells destined to become ameloblasts in the developing enamel organ were found to contain relatively large amounts of calcium and magnesium. During differentiation of the ameloblasts a marked diminution of salts occurred, so that the cytoplasm was found to be practically devoid of calcium and magnesium. This loss was accompanied, however, by an increase in other minerals, among which potassium was identified. In the fully differentiated ameloblast, just prior to the formation of enamel, the cytoplasm once again showed large quantities of calcium and magnesium concentrated within the distal and proximal areas of the cells. The central zone of the ameloblast was found to be relatively free from calcium and magnesium oxides. The mineral fluctuations observed in the differentiation and development of the enamel-producing cells were not observed in the odontoblast layer of the tooth germ. At every stage of their development the odontoblasts showed a constant mineral content. These cells were distinctly richer in calcium and magnesium when compared with the mesenchyme cells of the dental papilla. Hamp²⁸ found evidence for the assumption that the more mature the odontoblasts became, the greater was their magnesium and calcium content. He concluded from the evidence of these experiments that calcification takes place in alternating rhythms of high and low concentration and not in the form of a steady accumulation of inorganic salts.

k. Embryonic Tissues

Policard and Pillet⁷² first applied the micro-incineration technique to a study of the developing organism. Their examination was restricted to the calcareous deposits in the endolymphatic sacs of Anuran larvae. They detected appreciable amounts of calcium in the fluid of the cavities of the intracranial diverticula and the saccus endolymphaticus of the embryo. They contended that these deposits formed a reserve of calcium utilized during the development of the larva. Horning and Scott³⁹ studied the changes in the mineral distribution during certain phases of embryonic development in the chick. Apart from describing the ash organization of the developing organs, they found that embryonic development was expressed inorganically by an increase in certain mineral salts and a decrease in others. It was observed that in the brain the calcium salts were progressively increased, while iron was decreased. In the case of some other organs, during different stages of development, a definite migration of inorganic material had taken place. These results of Horning and Scott seem to indicate that mineral migrations take place during organogenesis as well as later, during the specific differentiation of cell types, as, for instance, in the enamel organ. Also, Alexander and Myerson³ found that the mineral ash in the human cerebral cortex was more concentrated during embryonic development than after differentiation had been attained. The poor concentration of iron oxide in the brain of the new-born infant was assumed to correspond with the lesser degree of vascularity of the new-born infant's brain. Their spectrographic examinations showed that the grey matter of the human adult brain was richer in iron, calcium, magnesium, and sodium than the same areas in the infant brain. The regions of the white matter were richer, however, only in phosphorus content.

V. PATHOLOGICAL CYTOLOGY

a. Arteriosclerosis

Arteriosclerosis is a disease which, generally speaking, increases in severity and frequency with advancing age. Since the time of Vesalius the existence of such lesions has been recorded, yet up to the present time there is no therapeutic treatment which will retard or cure the disease. Many theories of its pathogenesis have been evolved. Of the numerous papers on arteriosclerosis which have appeared during recent years, some of the most interesting are those which describe the pathological mineral changes as determined by use of the micro-incineration technique. Not only can the topographical position of the inorganic salts in question be examined but, as Policard⁶⁶ in his study of the subject points out, 'micro-incineration permits the investigator to determine accurately the very earliest changes in the mineral constituents at the

beginning of arteriosclerosis'. It is likely that the study of the very early lesions by micro-incineration will add considerably to our knowledge of the aetiology of the condition.

The haematoxylin staining of calcium deposits commonly employed by pathologists is, according to Cameron¹⁰, non-specific. Hence the micro-incineration technique has certain advantages over routine histological methods for the determination of calcium as well as iron and silica.

The manner in which calcium becomes concentrated within the walls of blood-vessels is a phenomenon not fully understood. It appears to be correlated with physical changes in the elastic tissue, as described by Wells^{114,115}. Ribbert⁸¹ was one of the first to show that perfectly normal arteries contain small quantities of calcareous material. Later, Faber¹⁹ found that calcium is chiefly located in the elastic laminae of old arteries, and is sometimes concentrated in the internal elastic laminae. Calcium deposition is rarely encountered in the intima or adventitia. Considerable doubt exists as to whether the calcifications sometimes seen in normal arteries represent an early phase in the aetiology of arteriosclerosis. Ribbert⁸¹ contends that such deposits bear no relation to the disease, while Faber¹⁹ regards them as an indication of the early onset of the condition. As most of these earlier observations have been based upon Kossa's method of silver impregnation, it is most likely that some of the controversial issues could be satisfactorily settled by studying the early phases of arteriosclerosis with micro-incineration technique. A systematic study of the topographical relationship between the mineral constituents in the arterial walls during the early phases of arteriosclerosis would most likely have a direct bearing upon the problem.

Some of the more important contributions in this field are those of Tschopp¹¹¹, Ravault^{78,79}, Policard, Morel, and Ravault⁷⁵, Ham²⁷, and Ku⁴⁴, all of whom have employed the micro-incineration technique for studying the pathogenesis of the disease. Ham's work on experimental calcification produced by large doses of irradiated ergosterol is most important, since it has brought to light fundamental changes in calcium deposition.

Tschopp¹¹¹ was the first to apply micro-incineration to a study of human arteriosclerosis in the uterus of a woman of only 46 years. He described the localization of calcium and iron salts in the arterial walls, and particularly the presence of large amounts of silica in the umbilical arteries of foetuses. He found most of the ash was located in the media, while the intima and the adventitia contained far less mineral residues. Ravault⁷⁹ gave the first detailed comparative account of ash distribution in histologically normal and diseased arteries. Calcareous deposits with iron oxide were located amongst the connective-tissue fibres and elastic tissues, but very rarely within the muscle-fibres themselves. In

healthy normal vessels the intima and adventitia yield no ash deposits, while calcium-containing ash in very small quantities was localized in the media. In calcareous deposits in the inflammatory foci of endarteritis a shift occurs in mineral salts from the media to the degenerating intima. Zinkant¹¹⁷ has confirmed these findings of Ravault in a description of increased ash deposits in the elastica interna of the uterine arteries in women of 50 years. As age advances an accumulation of inorganic salts, which were chiefly calcium, takes place in the media and frequently within the internal elastic membrane. Similarly, Policard, Morel, and Ravault⁷⁵, by using spectrographic analysis in combination with micro-incineration, were able to find the same sequence of changes in arteriosclerotic vessels in other parts of the body. Policard has also recommended the chemical analysis of the walls of large arteries about 1 mm. in thickness. By means of careful dissection the intima, media, and adventitia can be isolated and analysed separately by ordinary chemical methods. In view of more recent developments in the technique of micro-incineration, there appears little need to employ this tedious procedure, by which the topographical deposition of minerals can hardly be expressed in terms of cellular layers.

Ku⁴⁴ has reported a series of interesting observations on coronary sclerosis in a selected group of human cases, ranging from the new-born to 79 years of age. The smallest quantities of ash were found in the arteries of new-born infants and then, as age advanced, small increases in ash content were noted. This gradual increase in ash content was regarded as a normal phenomenon and not necessarily an early indication of the onset of arteriosclerosis. The ashed remains of the coronary arteries were found to consist almost entirely of calcium salts, which were localized mainly within the elastic tissue. Much smaller quantities were detected in the other tissues of the intima, media, and adventitia. The manner in which calcium salts are bound to elastic fibres was not determined by Ku. This author also found that as arteriosclerosis develops there is an increase in the amount of elastic tissue which was always accompanied by a corresponding increase in calcium ash. He concluded by suggesting that the increase of the elastic tissue has a direct bearing upon the calcification which occurs subsequently during the development of the disease. Ku also reported upon the variable increases of mineral constituents found in rheumatic fever nodules and in leukaemic infiltration.

Ham²⁷, in a study of experimental calcification, employed the micro-incineration method with great advantage in order to observe rapid changes in calcium deposition following treatment with irradiated ergosterol. It was found that large single doses of this substance produced massive calcification in the aorta, coronary vessels, and cardiac musculature of rats as soon as 48 hours following administration. Incinerated

sections of normal aorta from the rat and from material taken 24 hours after the administration of a single dose of irradiated ergosterol showed that the elastic fibres were devoid of mineral residues. In fact, detailed examination of sections 24 hours after treatment with ergosterol gave no indication of the high degree of calcification which was about to take place during the following 24 hours. The marked increase in the mineral content of the lesions produced 48 hours after a single dose of activated ergosterol were remarkably well shown in the incinerated remains of sections through the walls of the aorta and coronary vessels. The arteries stood out as thick-walled tubes of mineral residues when compared with the incinerated sections of similar vessels taken from material 24 hours earlier. At 48 hours after treatment the elastic fibres clearly became involved in the process, since they were heavily infiltrated with calcium-containing ash. Ham's experiments showed that the calcification was not dependent upon degenerative changes in the tissues affected. Judging from the rapidity and massiveness of these calcifications, he concluded that the phenomenon was related to the inability of the blood-plasma to retain all its calcium in solution.

Schultz-Brauns⁸⁹ and Schultz-Brauns and Schoenholz⁹⁰ consider abnormal deposits of calcium in cells to be due to a decreased functional metabolism probably associated with cell damage. Wehrefritz¹¹³ and Schoenig⁸⁷ hold the view that the presence of abnormal amounts of calcium indicates a physiological storage of that substance. Policard, Morel, and Ravault⁷⁵, in their spectroscopic studies of the minerals in blood-vessels, found that calcium and magnesium were present in small amounts in both normal and pathological vessels. The normal intima contained more calcium than magnesium, and the media was even richer in calcium and contained more magnesium than the intima. During lipoid degeneration, according to these authors, the intima shows an increased magnesium content, while in calcified atheromata there is a marked increase in calcium and not in magnesium. It was reported that all pathological mineral deposits of the aorta are calcareous, and that these conditions are not accompanied by a corresponding increase in magnesium salts. Unfortunately, these authors fail to mention the relationship between the elastic tissue and calcification.

b. Anthracosis and Silicosis

These pathological conditions have been investigated by Policard and Doubrow⁶⁹ and by Policard⁶⁵. For the quantitative estimation of the amounts of silica in silicotic nodules, the technique of micro-incineration has obvious advantages. These authors have noted in addition that the iron content of old haemorrhagic lesions in the lung could be demonstrated in ash preparations. Lecloux⁴⁵, when examining tubercular miners for pulmonary anthracosis, found that the dust cells in their

sputum contained an excessive amount of mineral particles after incineration.

c. Cerebral Lesions

Cerebral lesions of various kinds have been examined by the micro-incineration technique by Alexander and Myerson². According to these investigators, the inflammatory lesions of syphilitic encephalitis and dementia paralytica are all characterized by a state of hypermineralization. The ash accumulates in the nuclei and particularly in nuclear fragments, as well as in the degenerated remains of the infiltrative cells. The cytoplasm of these cells, especially those of glial origin, is much richer in calcium-containing ash than that of normal cells of similar type, while the infiltrative cells in neurosyphilis are extremely rich in iron oxide. Alexander and Myerson also found that the senile plaques in senile dementia and in Alzheimer's disease yield little or no ash after micro-incineration. A difference was noted in the ash of the neurofibrillar strands in Alzheimer's disease, namely, an abnormal amount of calcium-containing ash which was soluble, for the most part, in water. Later, these authors made an extensive study of the changes in mineral constituents of pathological brain tissue by combining the techniques of spectroscopy and micro-incineration. It was found that oedematous brain tissue was richer than normal brain tissue in both sodium and calcium, and that this increase was relatively greater in the white than in the grey matter. In dementia paralytica there was an increase of all mineral substances, which was accompanied by a decrease in the iron content of the total brain tissue. It was assumed that this finding indicated that loss of iron was caused by a decrease in the volume of the vascular bed due to a narrowing and obliteration of cerebral vessels, especially the capillaries. In studying cases of lead encephalitis Alexander and Myerson located excessive amounts of lead, which were concentrated in the grey matter rather than in the white matter of the brain.

d. Inclusion Bodies

Routine techniques have yielded as yet little data concerning the chemical nature of the intracellular 'inclusion bodies' caused by the selective action of certain filterable viruses upon animal tissues. Although much emphasis has been placed upon their morphology and staining reactions, the examination of their inorganic composition has given some valuable information. Owing to the fact that most intranuclear inclusion bodies can be inorganically differentiated by micro-incineration from the ash of the nucleus, it has been shown conclusively that the inclusion bodies are neither artefacts nor simple pathological derivatives of normal nuclear structures. Scott⁹³ was the first to incinerate material containing inclusion bodies, and he found that the

large nuclear inclusions caused by the submaxillary virus in guinea-pigs were almost ash-free, while the peripheral chromatin and the nucleolus were abundant in mineral substances. Cowdry's observations¹⁵ on the intranuclear inclusions in the liver cells of monkeys infected with yellow fever showed that these structures contained very little ash, and that they could be readily differentiated by their mineral organization from both the nucleolus and the chromatin. Covell and Danks¹⁴ reported that the cytoplasmic inclusions of rabies consist of a compact inorganic residue of white calcium ash. The Borrel bodies of fowl-pox were found by Danks¹⁸ to leave an abundant and characteristic mineral residue, composed largely of calcium. He concluded that the relatively large deposits of inorganic material in these structures served as a focus for the adsorption of the virus.

Rector and Rector⁸⁰ have demonstrated that the ash of the herpetic intranuclear inclusions in the cerebral cortex of rabbits varies according to the age of the inclusion body, the more mature inclusions containing appreciably less ash residue than those which were immature. In the advanced stages of the disease the inclusion body leaves little or no mineral residue, although the nuclei and chromatin still maintain a constant amount of mineral material, despite the striking changes in the cells resulting from the action of the virus. A similar differentiation between the ash resulting from the incineration of chromatin, nucleoli, and inclusion bodies has been demonstrated by Horning and Findlay³⁶ in cells infected by the Rift Valley fever virus. The residues forming the marginated chromatin consist mainly of calcium-containing ash. Findlay²⁰ had previously shown that these peripheral nuclear aggregations of chromatin were due to the action of the virus upon the cell. The nucleoplasm was devoid of ash, while the nucleolus formed a more compact mass of inorganic material, with a distinct yellow tinge suggesting the presence of iron oxide. The intranuclear inclusion was represented by a finely marginated inorganic residue, which was typical in appearance of the less mature inclusions of Herpes simplex described by Rector and Rector⁸⁰. No diminution of ash was observed in the inclusion bodies of Rift Valley fever such as these authors recorded in the herpetic inclusions. Nor was any visible increase in ash content of the inclusion body detected during acute liver necrosis, although this was shown to be associated with a marked increase and clumping of calcium salts in the cytoplasm of liver cells. The ash distribution resulting from liver necrosis produced by the virus of Rift Valley fever was compared with the ash residue of liver from uninfected animals in which necrosis had been produced experimentally by carbon tetrachloride poisoning, and in malignancy. Horning and Findlay found that the necrosis produced by these agencies, as well as that obtained with several other types of virus infection, led to a marked increase of calcium-containing

ash in the liver cells, followed by a final disappearance of the inorganic residues accompanying cellular degeneration. The final liberation of the inclusion body from the disintegrating nucleus did not influence the incineration picture in the case of material infected with Rift Valley fever. The mineral differentiation between the nucleolus and the inclusion bodies should help to settle the status of those inclusions which, from the evidence of staining reaction, are thought to be of nuclear origin.

e. Neurodermatitis

MacCardle, Engman, and Engman⁵⁰ examined the mineral distribution of skin specimens from 83 normal healthy persons of varying ages, 33 patients suffering from disseminated neurodermatitis, and another 55 with inflammatory disease of the skin, by means of micro-incineration and spectrographic analysis.

They found marked variations in the distribution of inorganic salts in the skin of healthy individuals, and were able to correlate this fluctuation with the age factor. The epidermal cells of normal skin are abundant in calcium and magnesium. The nucleoli of these cells consist of silicon. A hypomineralization is seen in the skin of healthy children up to the age of 10 years, after which a pronounced hypermineralization occurs and proceeds slowly until old age is attained. The primary mineral change which accompanies old age is a shift of calcium and magnesium salts from the deeper parts of the epidermis to the corneum and the dermis.

In incinerated active lesions of neurodermatitis skin, the spinous cells contain minute amounts of calcium and magnesium, but their nuclei are free from silicon. The ash residue of active lesions of localized neurodermatitis skin differs markedly from that of chronic disseminated neurodermatitis in that there occurs a mobilization of calcium and magnesium salts to the spinous cells, whilst chronic disseminated neurodermatitis, on the other hand, is accompanied by a loss of these elements. Incinerated sections of healed neurodermatitis lesions show the spinous cells rich in white calcium ash, and their nuclei are characterized by abundant silicon.

These authors contend that during the healing process the spinous cells regain their ability to retain biologically sufficient quantities of calcium, magnesium, and intra-nuclear silicon, and also that silicon is associated with the development and abatement of neurodermatitis lesions.

f. Pemphigus Lesions

MacCardle, Baumberger, and Herold⁴⁹ examined the skin of patients suffering from pemphigus lesions, and found that the outer third of the

dermis contained abundant silicon. The abnormal amount of silicon in the dermis appears to have been mobilized from the deeper dermis and to be deposited in large quantities as silicocalcareous compounds in the outer third of the dermis. They contend from the micro-incineration and spectrographic analytic studies that the mobilization of silicon and magnesium and calcium to the outer third of the dermis is a generalized phenomenon in pemphigus skin, not only in active lesions but also in the unaffected areas.

Incinerated pemphigus skin, regardless of age, shows that the dermis contains an excessive amount of silicon in the peripheral layer over that in normal skin. From these studies they contend that in pemphigus skin silicon is not only mobilized from one stratum of the dermis to another, as shown by micro-incineration, but is mobilized in the skin from the rest of the system, as revealed in the spectrogram by this increase in silicon in the whole skin. Also, they found that the iron content in cases of pemphigus was higher than in normal skin. Their calculations show that pemphigus skin contains twice as much iron as the normal healthy skin does. As shown by micro-incineration and spectroanalysis, a large proportion of this iron in pemphigus skin is located in the intercellular fibrils and in the cellular components of the connective tissue of the dermis. They contend that the excess iron in pemphigus skin is for the most part already in the form of an active, organically bound intracellular compound similar to cytochrome or Macallum's nucleoprotein iron compound. They conclude that it is probably active in some oxidative mechanism, and also suggest that pemphigus skin has a higher oxidative rate.

g. Neoplastic Growths

The question as to whether malignancy involves an increase or diminution in the mineral content of tissues was finally decided by the application of the micro-incineration method. The localization and orientation of the inorganic salts in normal and neoplastic cells *in situ* has been investigated by many workers. Policard and Doubrow⁶⁸ first examined tumour tissues by the micro-incineration technique and were impressed by the fact that cancerous tissues remain carbonized longer than normal tissues. Scott and Horning¹⁰² studied human medullary duct carcinomata of the breast together with several examples of scirrhous types. It was shown that the infiltrating new growths were characterized by a heavy deposition of mineral salts, and in comparison with similar normal tissues they were found to contain a relatively greater amount of mineral ash, especially of calcium and iron oxides. The nuclei of the malignant cells were not only richer in ash but contained more iron than the nuclei of normal healthy duct cells. The nuclear ash was concentrated along the nuclear membrane.

Further investigations on the mineral organization of a series of human neoplastic growths have been made by Olch⁶⁰ and by Olch and Scott⁶¹, in which they likewise described differences in the ash distribution between normal and malignant growths. Human naevi and hyperkeratoses were reported to possess a bluish ash in the areas of epithelial proliferation, which probably indicated an increased content of sodium. These authors made no mention of the presence of iron oxide in the incinerated sections of naevi. Horning and Lamb³⁷ examined human melanomata arising from the skin and from the choroid coat of the eyeball. The pigment of the melanotic sarcomata of the eye yielded an iron-containing residue, while the numerous pigmented naevi were optically devoid of iron. This was of interest, since the opinion is generally held that all melanomata contain variable amounts of free iron pigment.

Hamp²⁹ studied by micro-incineration an ameloblastoma, a slow-growing tumour of the jaw. Histologically these neoplasms closely resemble that of the developing enamel organ, but it is generally contended that they are incapable of forming enamel. By examining the quantity, quality, and distribution of the mineral deposits of this tumour he was able to come to the conclusion that the neoplastic cell is capable of forming enamel.

Alexander and Myerson² examined tumours of the brain, gliomatous as well as metastatic varieties, and confirmed the findings of Scott and Horning¹⁰², especially with regard to the increased nuclear accumulations of iron-containing ash in tumour cells. They also reported that all tumour cells were extremely rich in mineral substances when compared with normal brain cells. It was further found that most of the increased white cytoplasmic ash and the yellow nuclear ash of malignant brain cells were insoluble in water. These authors concluded that the increased mineral content of tumour cells might possibly play a role in X-ray sensitivity. Alexander and Myerson³, in a later communication, confirmed certain of their findings by using spectrographic analysis with micro-incineration. They found that the ash of meningiomata was 10 times richer in calcium than the normal grey matter, while other mineral substances were diminished in concentration. The mineral ash of a spongioblastoma of a mixed type, with a good deal of protoplasmic astrocyte differentiation, showed more potassium but less magnesium and phosphorus than normal grey matter.

Micro-incineration studies on tar tumours of rodents have also been described by Horning³³. Detailed comparisons were recorded between the ash of the hypertrophied tarred skin in mice and that of the underlying carcinoma cells in their varying degrees of differentiation. Variations in the mineral residues which accompany the skin hyperplasia and the subsequent development of tumour cells were observed. An increase of calcium ash was found consistently in the malignant and also in the

hypertrophied stroma cells (see Plate 5, Figs. 1 and 2). In this respect the tar tumours stand in contrast to those previously recorded, in which an increase of ash was found only in the malignant cells. The changes in the inorganic content of the epidermis during the process of keratinization were observed. An increase in calcium ash in the cell nests involved in this process leads to an obliteration of all cellular individuality in the ash. The iron-containing yellow ash of keratin can be traced amongst the flat white calcium ash of the disintegrating necrotic tumour cells. The fully formed keratin pearls consist of a series of concentrically orientated residues of deep yellow ash.

Cowdry¹⁶ examined the mineral changes occurring during skin hyperplasia in mice induced by treatment with 20-methylcholanthrene. In a 40-day hyperplasia, white ash, consisting of calcium and magnesium, was abundant and evenly distributed throughout the cytoplasm. Changes in the mineral distribution of the ash became apparent in specimens of skin 50 days after treatment, the mineral constituents becoming more concentrated in the stratum corneum and reduced in the stratum spinosum, whilst no changes above normal appeared in the epithelial pegs. As hyperplasia progressed, Cowdry reports a decrease in mineral salts in the spinous cells, which was characterized by a retention of ash in the single layer of the basal cells. In some pre-cancerous skin lesions hypomineralization of the spinous cells becomes more widespread and uniform. Cowdry also reported a correlation between white nuclear calcium ash in pre-cancerous skin lesion and thymonucleic acid. Scott¹⁰⁰ contends that malignant cells reveal changes in their mineral distribution unlike their normal prototypes. He states that cells forming epithelial proliferations in chronic cystic mastitis, and in human skin and breast cancer, possess less calcium deposits. Apparently the amounts of calcium ash present are dependent upon the pathological condition of the individual tumour cells at the time of incineration. In actively growing tumours, cells undergoing degenerative changes contain more calcium and magnesium residue in their cytoplasm than do younger and healthier cells in the same neoplasm. Healthier and actively growing malignant cells are frequently characterized by a hypomineralization. This difference in mineral content between healthy, actively proliferating and older, degenerating malignant cells comprising the same growth is of interest, and is a field in which more work with this technique should be carried out.

A relationship between the ash content of the nuclei of tumour cells and radio-sensitivity has been suggested by Cathie and Davson¹³. They claim to have demonstrated that the degree of radio-sensitivity of tumour cells is dependent upon the amount and arrangement of the nuclear inorganic material within the malignant cells. In support of their contention they quote the work of Lieber⁴⁶, who has shown that

the more malignant the tumour cell the greater the potassium/calcium ratio. The results of Cathie and Davson show that there are differences in the amounts and distribution of the nuclear ash between the less radio-sensitive squamous-celled carcinomata and the more radio-sensitive basal-celled tumours. These types of tumours were selected because they showed, within reasonable limits, a constant difference of sensitivity to radiation. The radio-sensitive basal-celled epitheliomata were found to possess a larger nuclear ash content than the less radio-sensitive tumour cells. Later, Cathie¹² introduced the photo-electric method for obtaining more accurate comparative measurements of nuclear ash differences. Nineteen cases of human new growths, of which the radiation lethal dosage was accurately known, were selected for these experiments. These results confirmed the former findings of Cathie and Davson that the inorganic nuclear ash could be employed as an index of tumour radio-sensitivity.

The action of radium radiations upon the inorganic structure of tumour cells has been investigated by Horning³⁴. Incinerated sections of adeno-carcinoma 27, a transplantable mouse tumour, were examined after periods ranging from 4 hours to 24 days following radium application. An increase in white calcium ash was detected as soon as 4 to 6 hours after radiation. This phenomenon was maintained in the cytoplasm 24 hours after radium treatment, and was accompanied by a marked cellular hypertrophy on the 3rd day. The maximum increase of mineral salts occurred on the 6th day following irradiation, at which time degenerative areas were found in all tumour-cell masses. The final disintegration phases of the dying tumour cells were characterized by alterations in the inorganic constituents in the form of a marked increase in calcium-containing ash. When reviewing these results it must be considered whether the increase in mineral salts, so conspicuous in tumour cells as soon as 4 to 5 hours after radium treatment, is due to the immediate action of the rays or to a secondary phenomenon following cell degeneration. In this respect it is interesting to note that Ludford⁴⁸, using a similar radium applicator for the same period, recorded definite cytological changes in the malignant cells of adeno-carcinoma 27 after 40 minutes irradiation. It might therefore be concluded that the increase in ash observed after 4 hours' irradiation was the result of a direct action upon the cancer cell. The maximum increase in calcium-containing ash, detected 5 to 6 days after radium treatment, was apparently due to a combined effect of the direct action of the rays, together with an indirect effect upon the stroma and tumour parenchyma, previously described by Bashford, Murray, and Cramer and Cramer⁵ and Cramer¹⁷. The gradual and extensive development of the stroma and the destruction of the blood-vessels influenced by radium treatment would obviously lead to degeneration and necrosis. These aspects of the problem were

clearly revealed by the micro-incineration process. The marked increase in calcium ash in necrotic areas of the irradiated tumours was similar to that previously reported by Scott and Horning¹⁰² in human duct carcinoma of the breast.

At present it is difficult to explain these findings, especially the increase of calcium ash in tumour cells following short intervals after radiation, but it seems obvious that any increase in calcium can only come from the blood-stream, in which case the radium radiations might affect the permeability of the cell membranes in such a manner as to allow an increased penetration of calcium ions into the malignant cells.

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FIG. 1. Photograph of electric quartz incinerator. A = ammeter, B = door, used for regulating current of air, Q = quartz tube surrounded by heating element, S = silica slabs on which the glass slides bearing the sections rest inside the furnace, R = rheostat by which the temperature of the apparatus is controlled

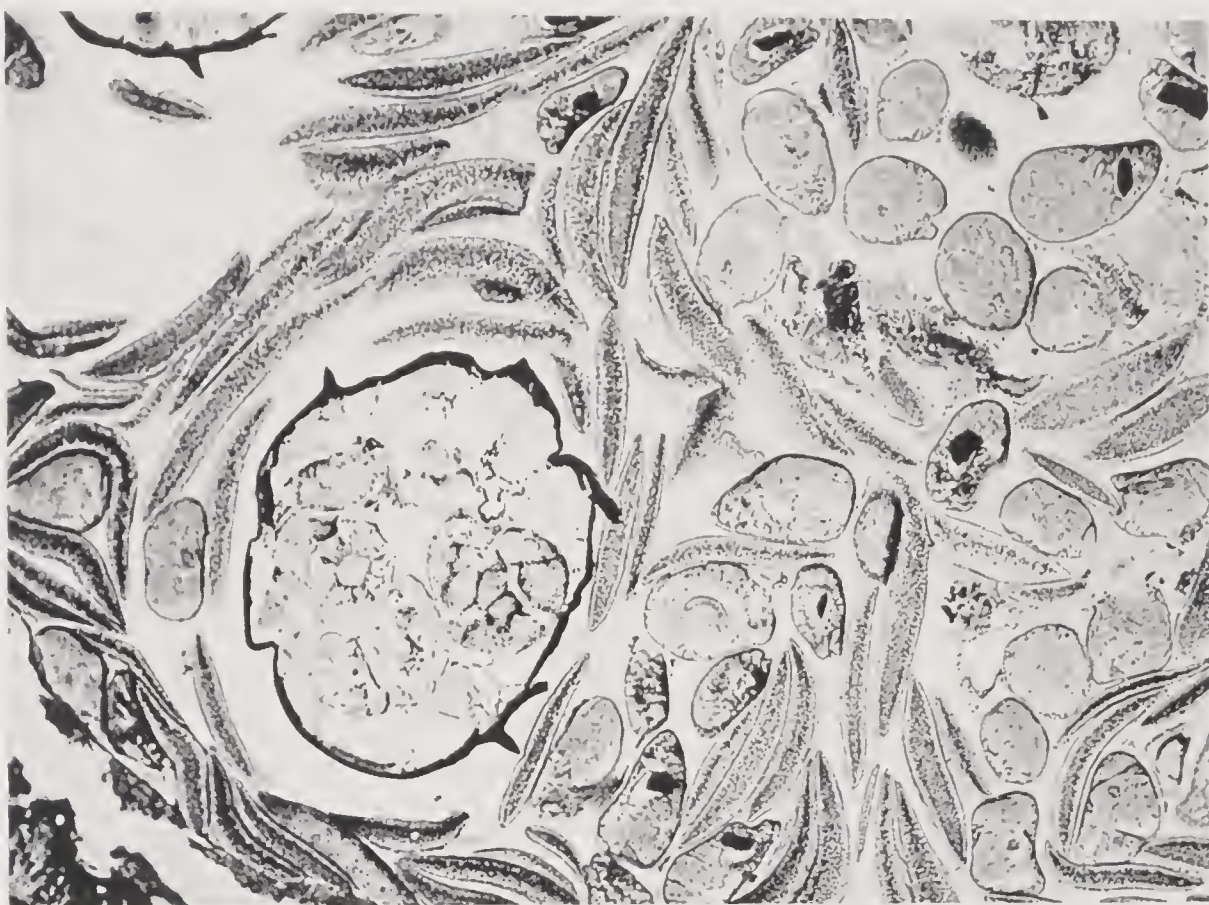


FIG. 1. Photomicrograph of control section, cut through the rectum of *Rana pipiens*, infected with *Opalinids* and *Nyctotherus cordiformis*. The vegetative granules in the elongated *Opalinids* and the macronuclei in the *Nyctotherus* are clearly shown

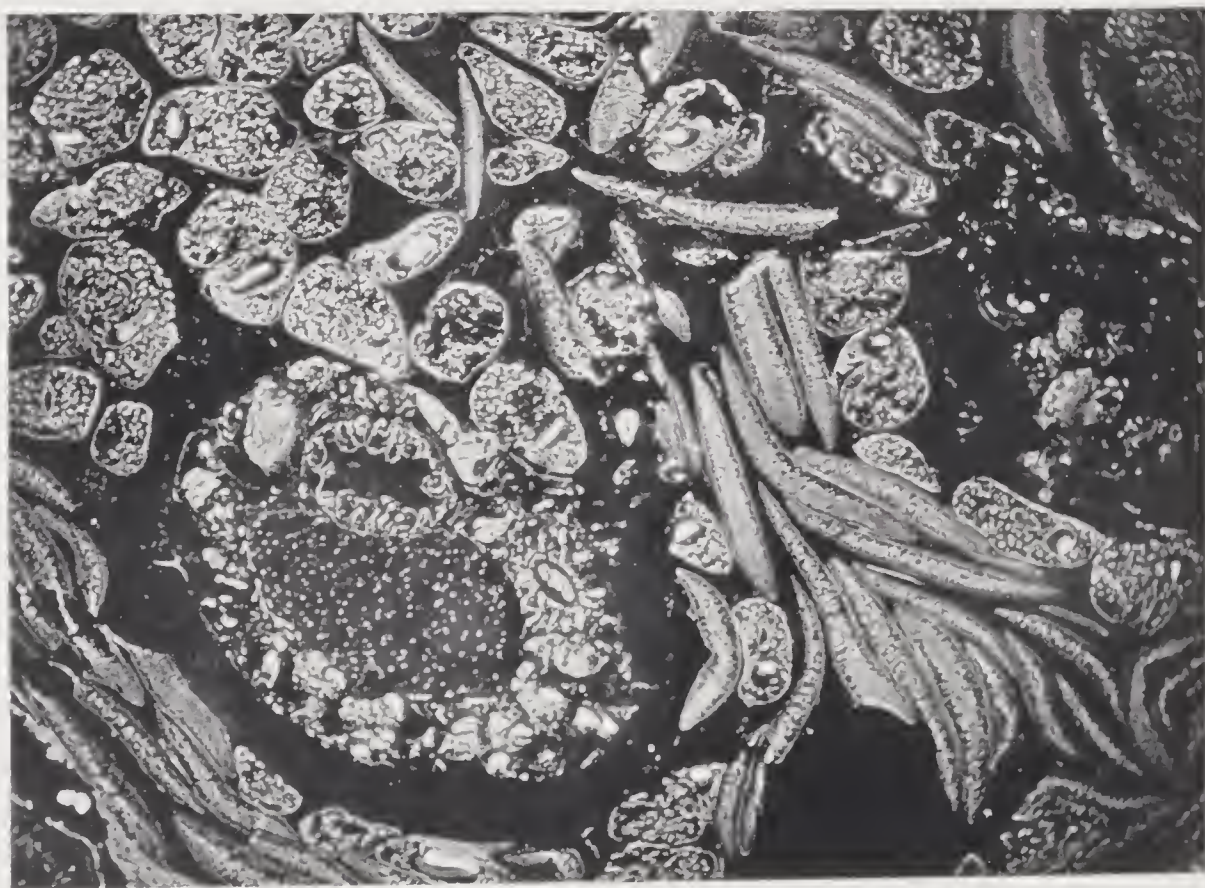


FIG. 2. Incinerated section, similar to the above, photographed by dark-field illumination. The vegetative granules leave residues of white calcium-containing ash

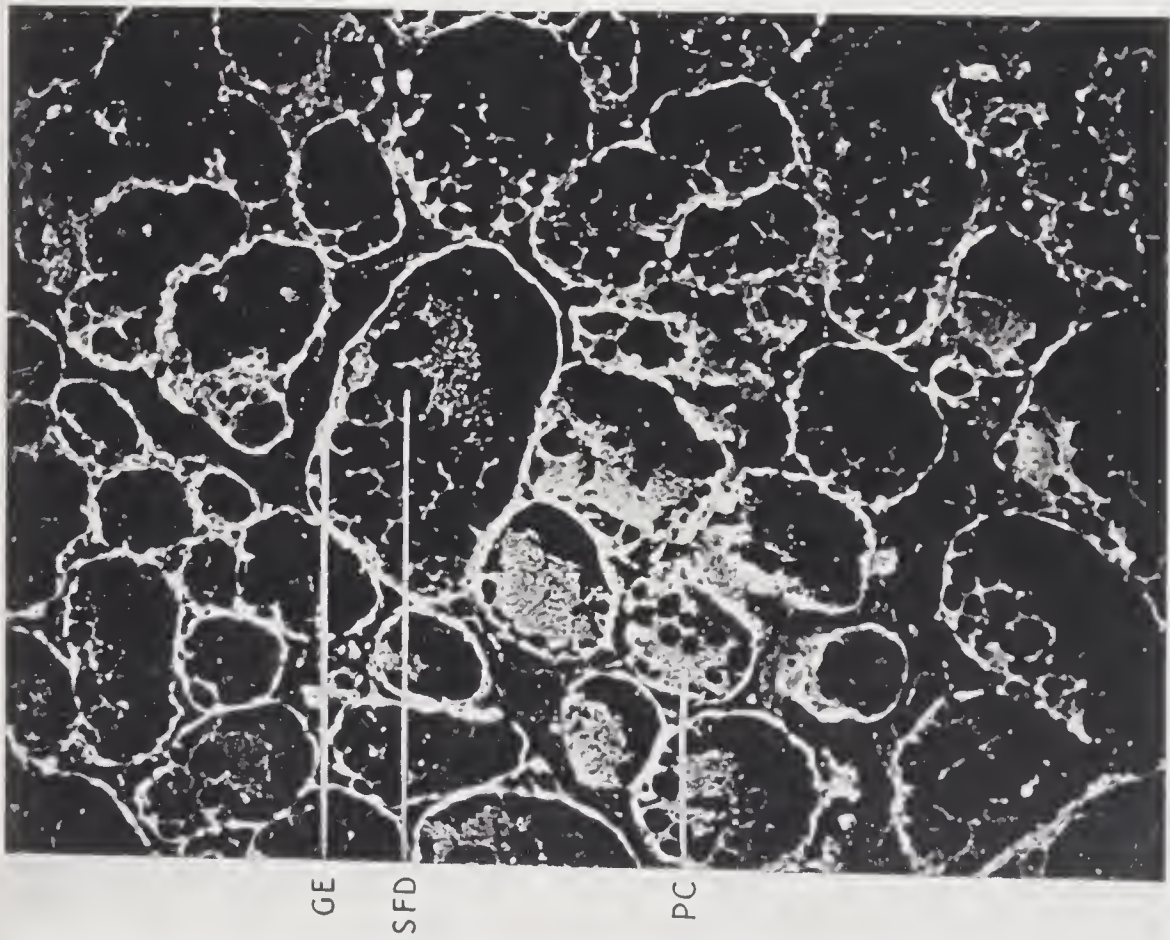


FIG. 1. Photomicrograph of an incinerated section from an un-irradiated lactating mammary gland of a mouse (dark-field illumination)

PC = protein coagulum.
GE = glandular epithelial cell.

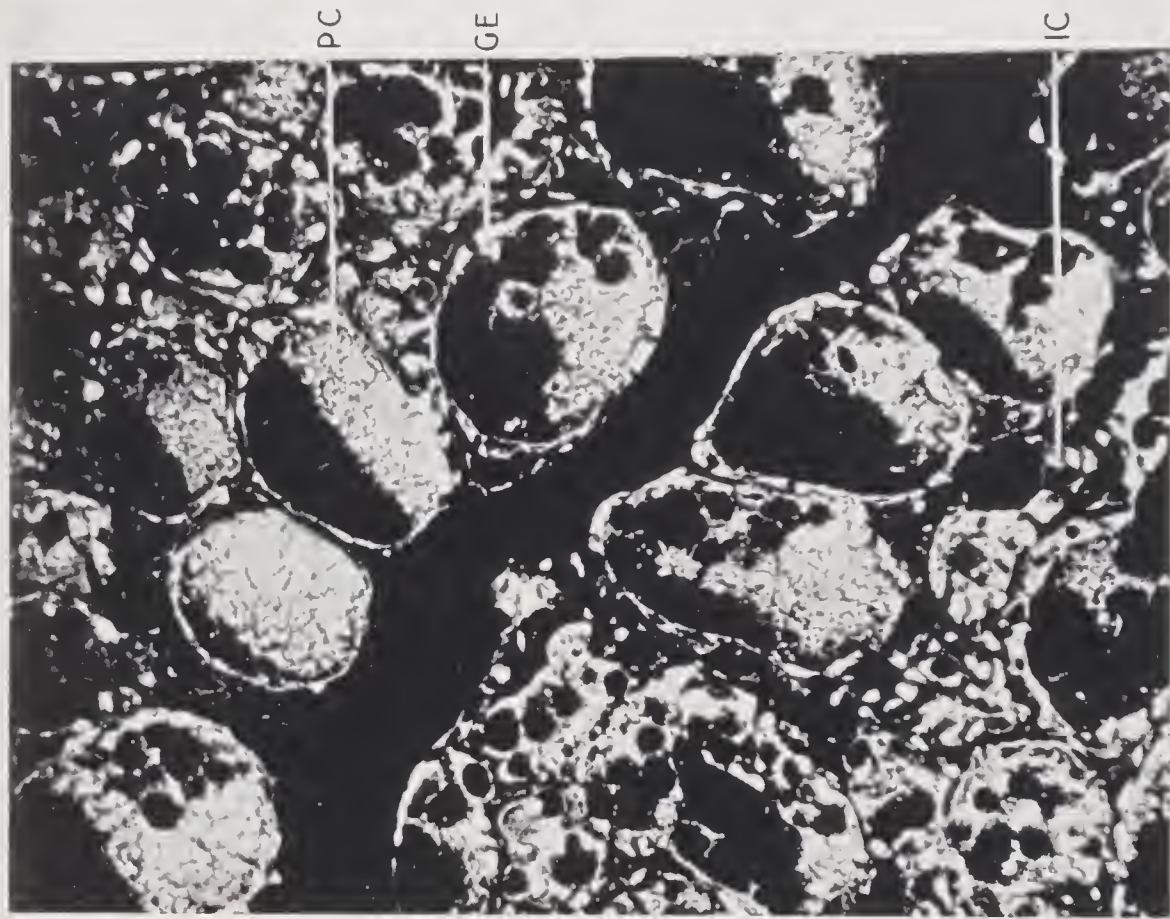


FIG. 2. Photomicrograph of an irradiated lactating mouse mammary gland, taken in dark field after micro-incineration. A marked increase is shown in the inorganic salts of the milk, after irradiation

IC = Intralobular connective tissue.
SFD = Space once occupied by fat.

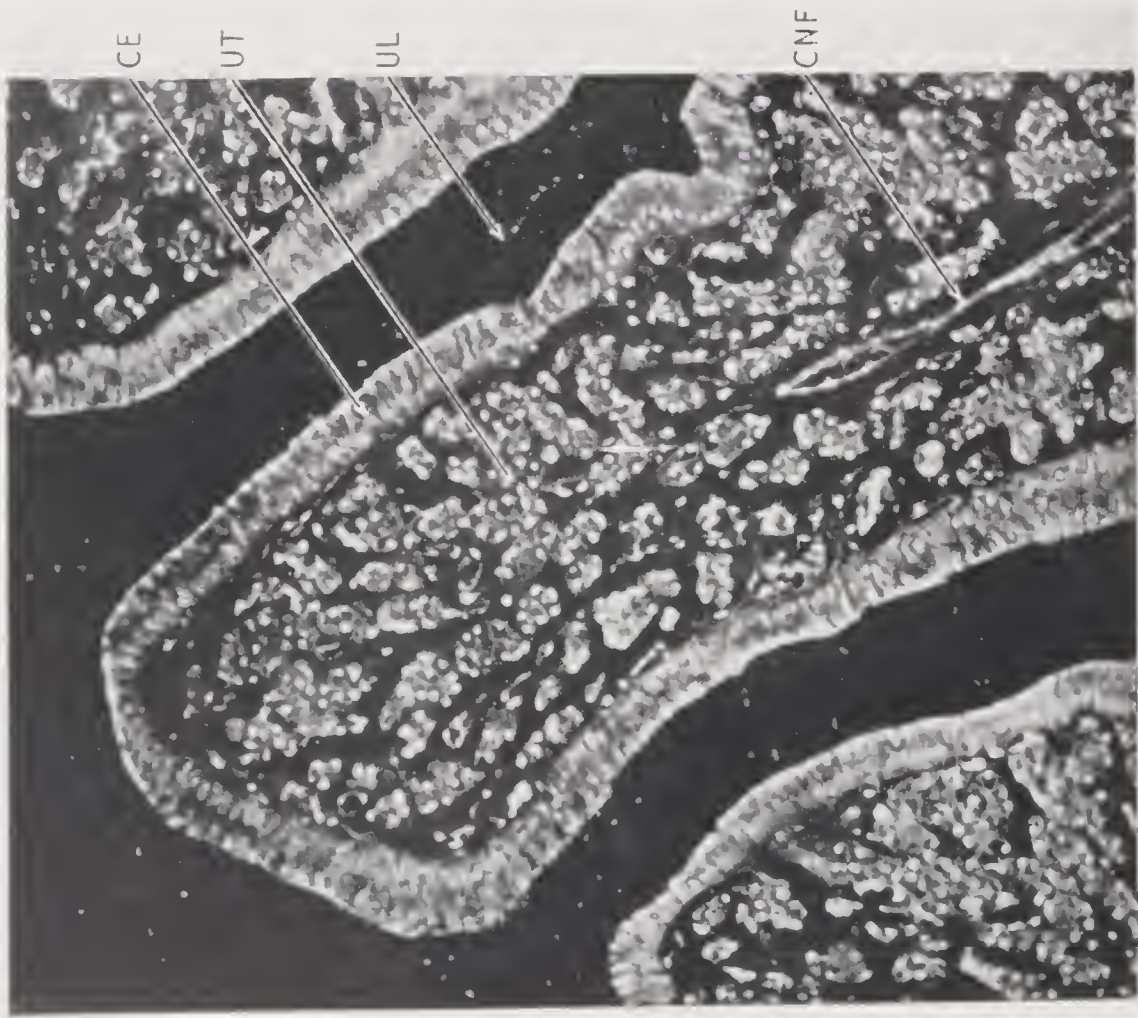


FIG. 1. Portion of the uterine mucosa taken from a domestic hen at a time when the uterus contained a soft, partly calcified egg-shell. There is a heavy concentration of white ash in the incinerated section, which is localized particularly in the surface epithelium. $\times 175$
CNF = Connective tissue axis of fold. UL = Uterine lumen. UT = Uterine gland.
CE = Ciliated epithelium.

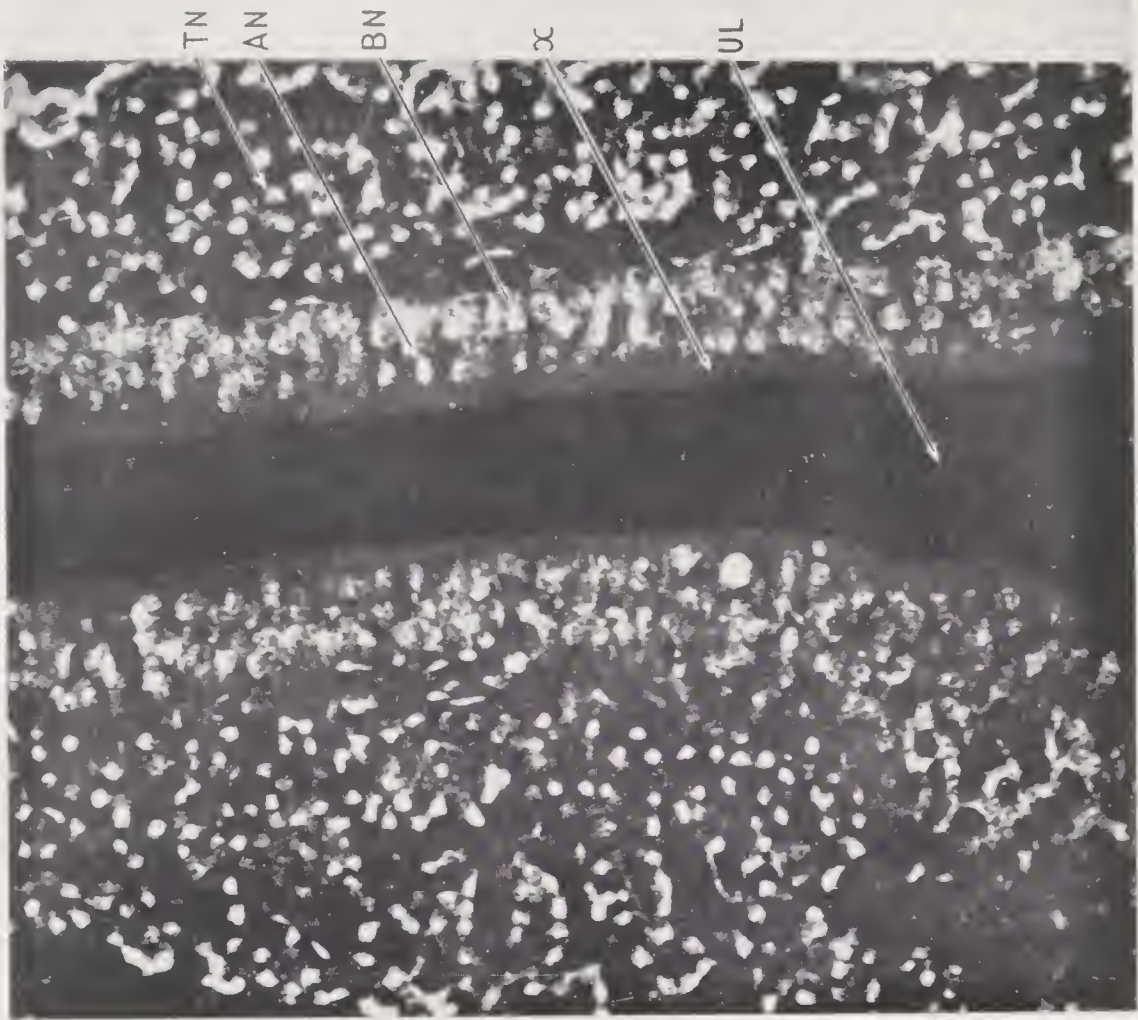


FIG. 2. A similar area of uterus, under higher magnification, from a fowl which had laid an egg about 4 hours previously. The lining epithelium of the empty uterus is conspicuously free from ash residues except for the nuclear zones. $\times 300$
x is used by the author (K. C. Richardson) to indicate special points in the figure referred to in his text. UL = Uterine lumen. BN = Basal epithelial nucleus. AN = Apical epithelial nucleus. TN = Gland epithelial nucleus.

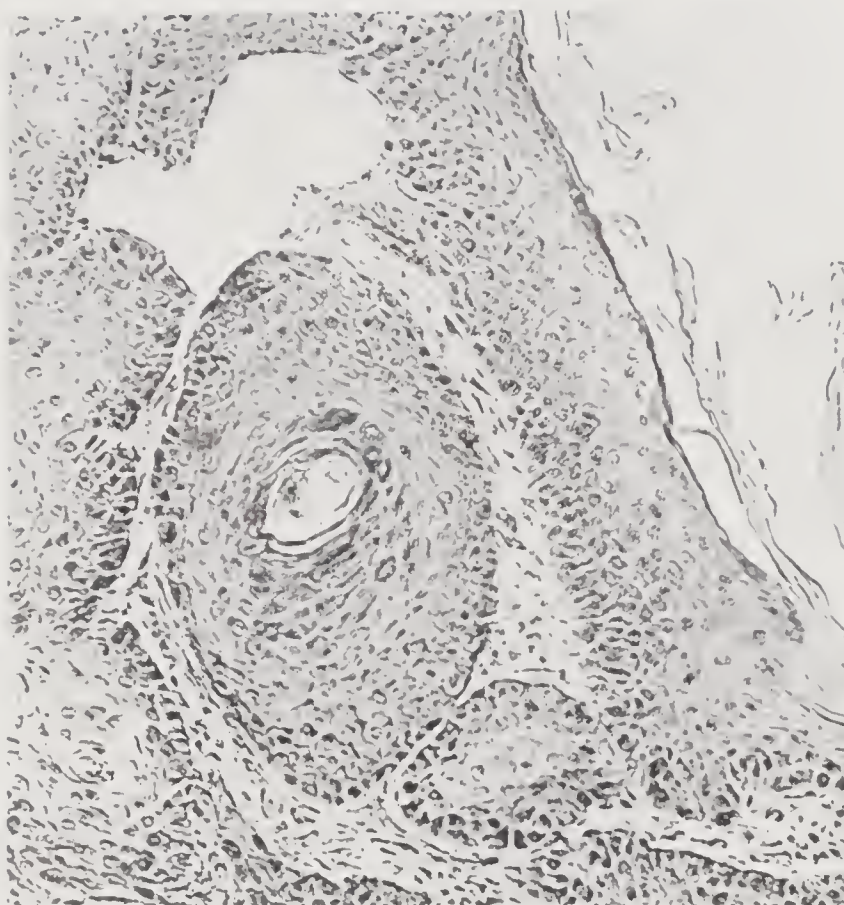


FIG. 1. Photomicrograph by direct illumination of a control section of a mouse tar tumour, stained with Erhlich's haematoxylin, after fixation in alcohol-formalin, showing the tarred skin, the stroma, and the underlying tumour cells

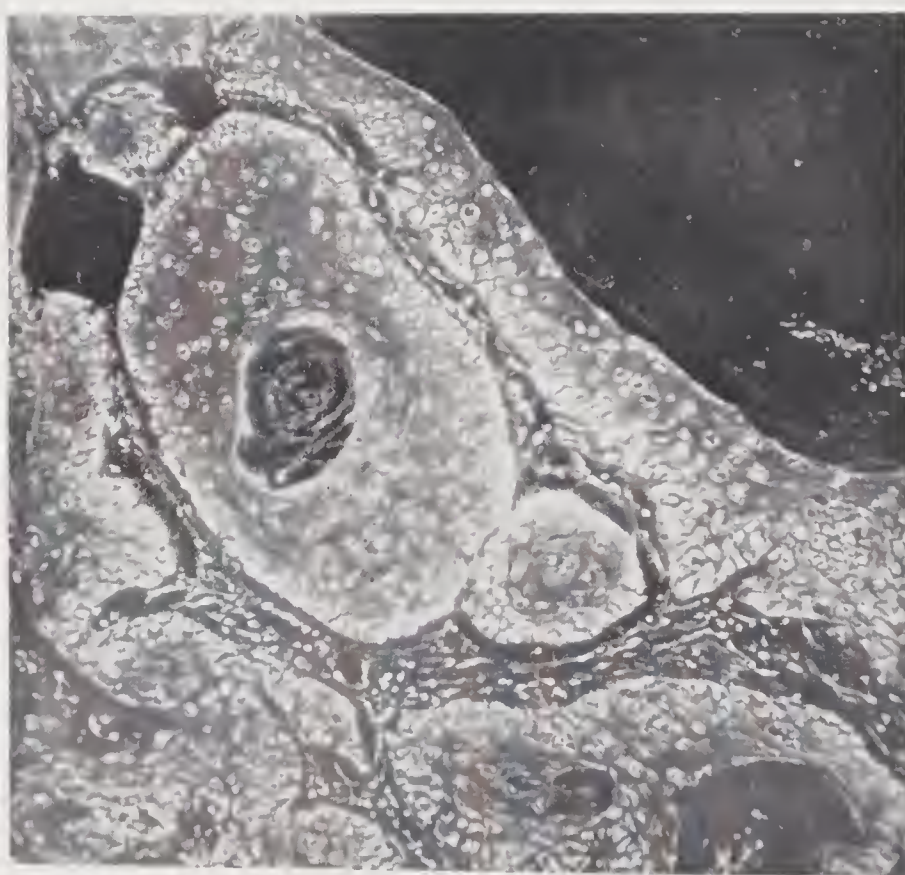


FIG. 2. Incinerated section, similar to above, photographed by dark-field illumination

E. S. Horning

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CHAPTER VIII

ENZYME SYSTEMS OF CELLS

By H. BLASCHKO *and* W. JACOBSON

Revised by H. BLASCHKO *and* F. K. SANDERS

INCREASED understanding of the metabolic reactions in cells has made the central position of intracellular enzymes increasingly clear. Cell metabolism, catalysed by intracellular enzymes, is the main theme of modern text-books of biochemistry (e.g. Baldwin¹⁰). Similarly, the structural features of the cell must depend on the presence of catalysts which regulate the synthesis of their component materials.

Specific differences between cells must therefore find their explanation in differences in the enzymic equipment. The enzymic equipment is an individual characteristic of the cell; both inheritance and environment will determine it. Enzyme formation is thus expected to be gene-controlled, but also to be dependent on other factors.

i. GENE CONTROL OF ENZYME FORMATIONS

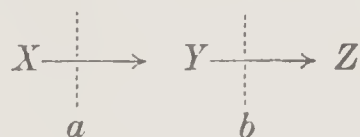
a. Micro-organisms

The study of genes in micro-organisms in connexion with the enzymic equipment has repeatedly been fully discussed; the reader is referred to the Symposium on heredity and variation in micro-organisms (²) and to the Conference on gene action in micro-organisms¹.

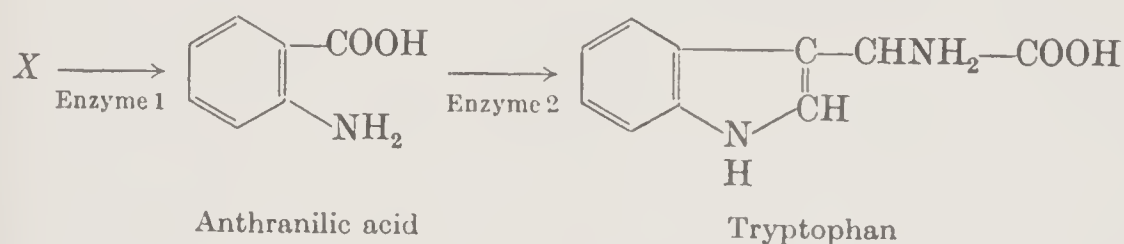
Gene mutations have been studied which manifest themselves in metabolic differences between parent organisms and offspring. Such metabolic mutants have been produced artificially, e.g. by radiation. Under these conditions cells may have been found to have arisen which differ from the parent strain; they will grow only in a medium which contains a substance not required by the parent strain. The fungus *Neurospora* is a particularly well-studied organism, as it is relatively easy to obtain and propagate the offspring from single cells (see review by Beadle¹³). Mutant strains of *Neurospora* have been produced which require one particular amino-acid (e.g. lysine or tryptophan or arginine) or one of the members of the B group of vitamins (e.g. pyridoxine). It is assumed that a mutant strain is unable to perform the synthesis of one substance which the parent strain can produce. The parent organism contains an enzyme essential for the synthesis; the mutant strain is deficient in this enzyme. The mutant of *Neurospora*, which requires the presence of pyridoxine, has been used for the assay of this substance: with low concentrations, the amount of pyridoxine added becomes the

limiting factor for growth; growth in the presence of an unknown amount of pyridoxine can be compared with growth in the presence of known amounts of added pyridoxine¹⁸⁹.

Deficient mutants are of interest in the study of intermediary metabolism, as they often give an indication as to the sequence of chemical reactions in the synthesis of a cell constituent. A substance *Z* may arise in the cell from an ultimate precursor *X* by a number of steps; in the example given, there are two steps, (*a*) and (*b*):



Each step will require its own specific catalyst. The chain of reactions leading from *X* to *Z* may be broken either at *a* or at *b*. If the catalyst responsible for step *a* is absent, substance *Y* will be able to replace *Z* in the nutrient medium. Substance *Y* will not be able to replace *Z* if the mutant strain is deficient in the enzyme required for step *b*. An example of this kind may be quoted from *Neurospora*, where two mutants exist which differ from the wild strain in their requirement for tryptophan. Both strains will grow when tryptophan is present in the medium. One of the strains will grow also when tryptophan is replaced by anthranilic acid, but with the other strain tryptophan cannot be replaced by anthranilic acid. It is therefore concluded that anthranilic acid is a normal precursor of tryptophan in *Neurospora*:

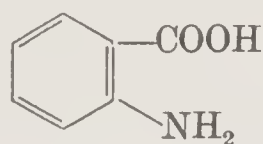


A strain deficient in enzyme 1 which is necessary for the synthesis of anthranilic acid will grow in the presence of either tryptophan or anthranilic acid; a strain deficient in enzyme 2 essential for the formation of tryptophan from anthranilic acid will not grow when tryptophan is replaced in the medium by anthranilic acid. Many examples of metabolic reactions could be quoted where the study of growth requirements in mutants has helped the biochemist to understand the sequence of reactions which occur in the bio-synthesis of a cell constituent.

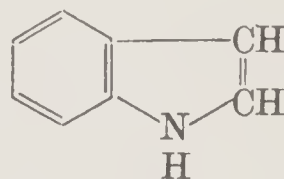
Deficient strains are interpreted as gene mutants, and this makes them of interest in the study of inheritance in micro-organisms. The first observation on gene recombination was made by Lederberg and Tatum¹²¹ in *Escherichia coli*. The authors used two strains of this organism, which showed multiple gene mutations. One strain had the following three deficiencies: it required the presence of thiamin,

threonine, and leucine; the other strain required these three substances: biotin, phenylalanine, and cysteine. Both strains were grown together, and from the mixed cultures a number of new deficient strains were isolated. Among these there were three strains which exhibited double deficiencies. One of these strains required both biotin and leucine, one required both biotin and threonine, and the third required both biotin and thiamin. Each of these strains combined deficiencies of which one was derived from each 'parent' strain. This experiment furnishes support for the occurrence in bacteria of gene recombination similar to that occurring in organisms which exhibit sexual reproduction.

The study of spontaneous mutations is facilitated in micro-organisms by the rapid succession of generations and by the ease with which large numbers of single organisms are obtained. Here, too, the study of growth requirements reveals enzymic or, more precisely, metabolic mutants. The mutant may differ from the parent organism in that it does not require a constituent of the medium which the parent organism requires ('non-exacting mutant') or the mutant may require a substance which the parent organism can synthesize ('exacting mutant'). For non-exacting mutants, selection is favoured when the organism is grown in a medium poor or deficient in a particular constituent. This process of selection is often referred to as 'training', because the organism appears to have been taught to do without this constituent of the medium. A well-studied instance is known for the organism which causes typhoid fever in man, *Eberthella typhosa*, which has been examined by Fildes and his colleagues⁶⁸. Most pathogenic strains of this organism are exacting towards the amino-acid tryptophan. By repeated subcultivation in media successively poorer in tryptophan strains were obtained which did not require tryptophan. The organism was said to have been trained to do without added tryptophan. The non-exacting strain differs from the parent organism in that it contains an enzyme essential in the formation of indole from anthranilic acid:



Anthranilic acid



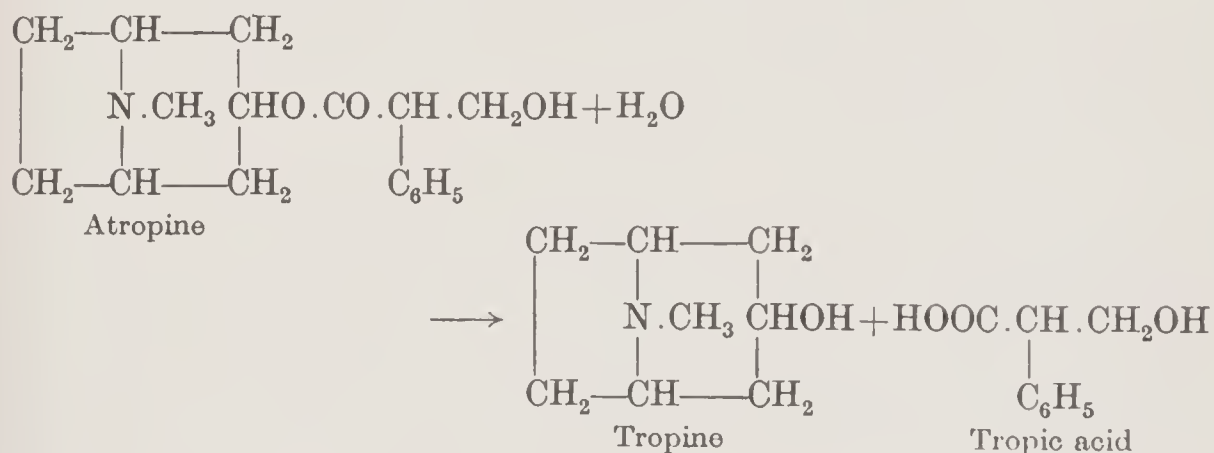
Indole

This reaction is believed to be one of the steps in the synthesis of tryptophan in this organism. Fildes and Whitaker⁶⁹ have recently shown that the appearance of non-exacting cells does not depend upon the environmental changes, but on the numbers of cells per generation. Non-exacting cells were found to be present in the ratio of one to 2×10^6 to 10^8 cells; the deficient medium facilitated the selective propagation of the non-exacting cells.

In the interpretation of the spontaneous variability of micro-organisms as gene mutations, the occurrence of a mutation is assumed as determined by the mutation rate, i.e. by the probability of a mutation per bacterium per generation (Luria¹⁴⁰).

b. Metazoa

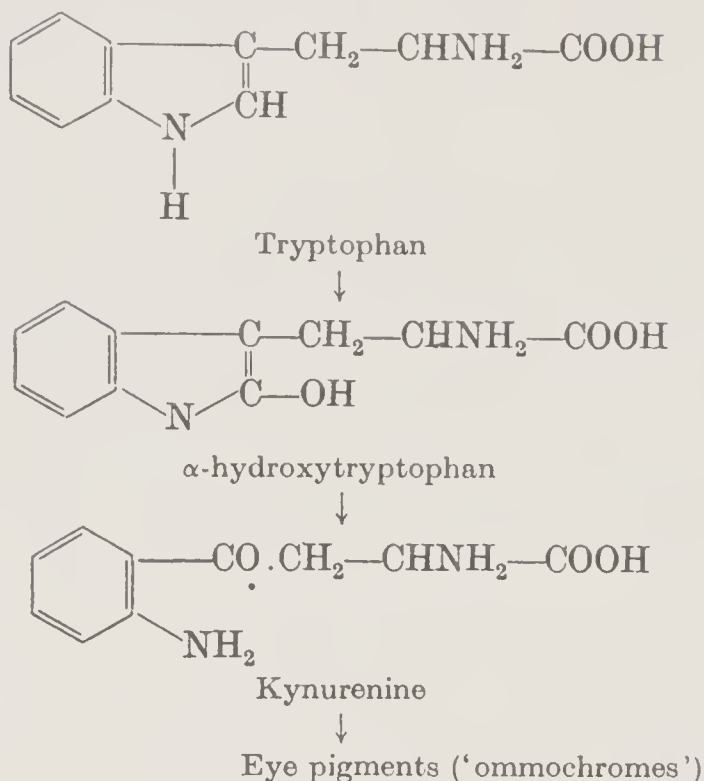
In higher organisms, individual differences in metabolic performance in a species are often interpreted as genetically controlled differences in enzyme formation. The enzymes involved are usually not known, but in some instances the inheritance of enzyme differences in a species has been studied. It has long been known that in the blood of some rabbits atropine is rapidly hydrolysed. This is due to the presence of an enzyme, tropinesterase, which catalyses the hydrolysis of atropine, with the formation of tropine and tropic acid:



It has been shown that the presence of this enzyme, which is present in about one out of twelve rabbits, is inherited as a Mendelian character¹⁸³.

The analysis of the control of pigment formation in the eyes of certain insects provides an instance where the reaction controlled by the gene seems to be established, although the enzyme is not known. In the meal moth *Ephestia kühniella* and in the fly *Drosophila melanogaster* there exist races which differ from the wild specimens in that the dark eye pigments present in the latter are not formed. These deficiencies are gene-controlled. It was shown by Kühn for *Ephestia* that in the larvae of the light-eyed mutant darkening of the eyes could be brought about by an implantation of tissue from dark-eyed specimens. Similarly, in the red-eyed race of *Drosophila*, called 'vermillion', the absent gene 'v⁺' could be replaced by a substance formed in the wild brown-eyed insect (see review by Beadle¹²). The substance absent in the deficient varieties was shown to be closely related to tryptophan. This v⁺-substance is probably identical with kynurenine, an amino-acid derived from tryptophan which is also found in mammals as a breakdown product of tryptophan. Its chemical constitution has been determined by Butenandt and his colleagues³⁴. It has also been shown that

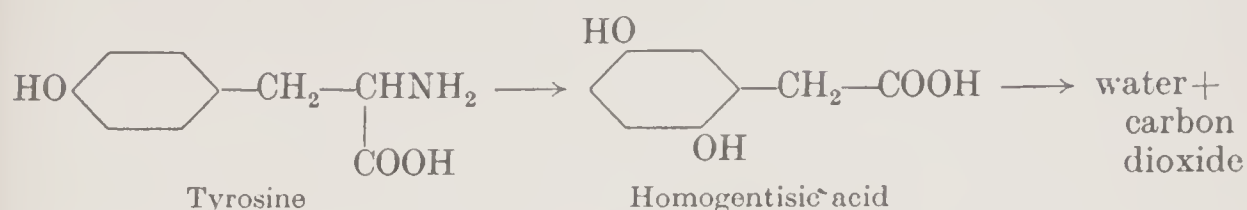
the amino-acid α -hydroxytryptophan will replace the v^+ -substance in *Drosophila*, and the following sequence of reactions has therefore been proposed:



Since α -hydroxytryptophan will replace the v^+ -substance it seems that the enzymic deficiency in the 'deficient' mutants of *Ephestia* and *Drosophila* is to be found in the absence of the enzyme which catalyses the oxidation of tryptophan to α -hydroxytryptophan. Such an enzyme is not known, but it seems possible that it is part of an enzyme system occurring in the mammalian liver and described as 'tryptophan pyrrolase' by Kotake and his colleagues¹¹⁴; it catalyzes the formation of kynurenine from tryptophan. L- α -hydroxytryptophan has been found in nature as a constituent of phalloidin, the poison of the toadstool *Amanita phalloides*; phalloidin is a polypeptide and α -hydroxytryptophan is one of its hydrolysis products²⁰².

The inherited metabolic disorders in man and other mammals present other instances of gene-controlled metabolic deficiencies often explained as enzymic deficiencies. Alcaptonuria in man is a much studied condition of this kind. In the urine of the alcaptonuric subjects a reducing substance is excreted which has been identified as homogentisic acid. In the affected subject, an increased intake of the amino-acid tyrosine is followed by an equivalent increase in the excretion of homogentisic acid. Administered to a normal subject, homogentisic acid is completely metabolized, but in the alcaptonuric subject it reappears quantitatively in the urine. On the basis of these facts, homogentisic acid is generally considered as a normal intermediate in the oxidative breakdown of tyrosine; alcaptonuria is interpreted as a condition in which the enzyme responsible for the oxidative removal of homogentisic acid

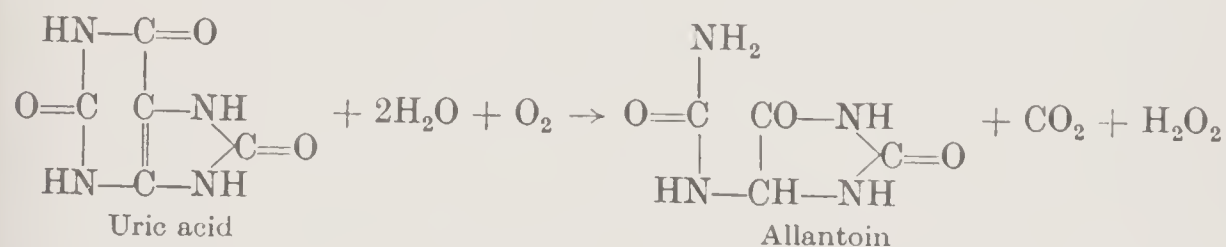
is absent⁷⁴. In other words, in alcaptonuria homogentisic acid appears as an end product of tyrosine metabolism.



Alcaptonuric subjects have been much in demand for the study of the intermediary metabolism of tyrosine: a compound suspected of being an intermediate on the way from tyrosine to homogentisic acid must be expected to be excreted as homogentisic acid in the alcaptonuric subject. In fact, there exist quite a number of substances which fulfil these conditions, and their rival claims to be considered as normal intermediary metabolites are still under discussion^{19,165,166}.

It is known that the deficiency in alcaptonuria is gene-controlled. The mode of breakdown of homogentisic acid in the normal subject, however, is not known, and the existence of an enzymic deficiency is therefore still hypothetical.

A gene-determined metabolic deficiency cannot always be easily explained by the absence of an enzyme. In the Dalmatian coach-hound uric acid is excreted as an end product of purine metabolism; in most other dogs allantoin takes the place of uric acid. The excretion of uric acid is inherited as a recessive character, controlled by a single gene¹⁹¹. Allantoin is formed from uric acid by the enzyme uricase which catalyses the reaction:



It might be expected that the Dalmatian dog is poor in uricase. This, however, is not the case: the enzyme is present,²⁰⁰ but this breed is unable to reabsorb uric acid in the renal tubules (for literature see Rimington¹⁷⁶).

ii. NON-GENETIC CONTROL OF ENZYME FORMATION

a. Adaptation in Micro-organisms

In bacteria and other micro-organisms many instances are known where formation of an enzyme is dependent on environmental conditions. Karström¹⁰⁴ first distinguished two types of enzyme, 'constitutive' and 'adaptive'. Constitutive enzymes are formed in the cell in the absence as well as in the presence of their substrates: adaptive enzymes are formed only when their specific substrates are present in the medium.

Examples of adaptive enzymes are the bacterial amino-acid decarboxylases and some of the enzymes concerned in the fermentation of carbohydrates (other than glucose) in yeasts. Adaptive enzymes are often found fully developed in the first generation of cells grown in a medium containing the specific substrate; conversely, enzymic activity disappears rapidly when growth occurs in the absence of substrate. This often serves as a criterion by which the process of adaptation is distinguished from 'training'^{53,73}.

It is assumed that the ability of a cell to form an adaptive enzyme is an inherent character of the cell and that it is gene-controlled; in the absence of substrate the amounts of enzyme are so small that they cannot usually be measured. This finds support in observations by Gale⁷² that in *Streptococcus faecalis* small but measurable amounts of L-tyrosine decarboxylase are formed in the absence of added substrate.

To what extent 'training' and adaptation are responsible for the changes of metabolic activities of cells is still under discussion. In particular, this is so in the phenomenon of drug resistance in micro-organisms, to which both adaptive processes in individual cells and selection of mutant strains contribute^{86,140,204}.

It has been claimed that in hybrids of *Saccharomyces* formation of adaptive enzymes will continue even in the absence of the genes necessary for enzyme formation¹⁸⁸. This is, however, not generally accepted, and the experimental basis of the 'cytogene' theory of enzyme formation has been criticized by Winge and Roberts²⁰³, who do not consider the occurrence of gene-independent enzyme formation as established.

b. Enzyme Equipment of Somatic Cells

In metazoa, the enzymic equipment of different cells of the same individual organism differs as widely as do the structural features. As in micro-organisms, it is the potential metabolic activity of the cells which is determined by the genes; their actual enzyme content is controlled by those factors which bring about cell differentiation. Very little is known about the mechanism by which these factors become effective. A classification of enzymes corresponding to that made for micro-organisms has not been made. It seems uncertain if enzymes could be found which are present in all cells of a given organism. Moreover, the influence of environmental factors on many somatic cells is not marked. If we assume that the gene equipment of all somatic cells in one organism is the same, it remains to be explained why a cell continues to 'breed true' in a culture, where environmental influences have been removed.

Normally, the enzyme equipment of a cell is probably determined during cell growth, but there are observations which indicate that this is not always true. The enzyme equipment of a cell can be increased

by implantation of similar tissue with a different enzyme content. Billingham and Medawar¹⁶ have studied the spread of pigmentation occurring after an implantation of autografts of pigmented skin in an area of white skin in the guinea-pig. This spread was traced to a spread of melanin formation from the melanoblasts to similar but non-pigmented dendritic cells of the white area of the skin. The melanoblasts gave a positive 'dopa-oxidase' reaction, but the non-pigmented dendritic cells were dopa-negative. In the region of pigment spread, however, the dendritic cells were transformed into melanoblasts and became dopa-positive. The authors interpret the spread as a spread of pigment formation; the process is likened to an infection carried by way of well-developed cytoplasmic connexions from the melanoblasts to the non-pigmented dendritic cells. The authors rule out the possibility that the spread is due to a diffusion of the pigment or of the pigment-forming enzyme.

In these experiments the pigmented cells as well as the non-pigmented cells came from the same animal; they were derived from cells with identical gene equipment. We have therefore to ask: Were the cells still genetically equivalent, and did the process of 'infection' consist in the release of the potential ability of the cell to form the enzyme, or had the non-pigmented cells lost the gene? According to the authors both pigmented and non-pigmented cells 'breed true'. Another question which cannot be answered at present is: What is the nature of the infecting agent?

iii. GENE-ENZYME RELATIONSHIP AND SPECIFICITY

Enzyme formation is probably a special case of protein formation; nuclear control of the latter has recently been discussed by Caspersson³⁵. Enzyme formation is not only determined by nuclear genes *sensu strictiori*, but also by related gene-like structures such as phage or virus ('naked genes', Muller¹⁵⁵). The best studied of these gene-like agents is the type-transforming principle of pneumococci described by Avery, McLeod, and McCarty⁸. Virulent pneumococci are characterized by the possession of a capsule composed of complex polysaccharides; the removal of the capsule renders the cocci non-virulent. Different types of capsular material can be distinguished by means of specific immune sera. Each of these type-specific capsular polysaccharides differs from the other in its chemical composition. Type specificity is an 'inherited character'. From encapsulated pneumococci of type III a fraction has been obtained which conferred on non-encapsulated pneumococci of other types the ability to produce the capsular polysaccharide of type III; the cocci thus obtained showed all the properties of type III pneumococci; type specificity in these cells had become an acquired inherited character.

The type-transforming principle is not identical with the capsular polysaccharide; it has been purified by removing:

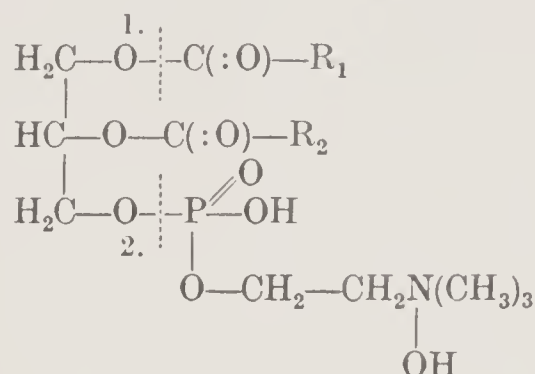
- (a) proteins by chemical methods,
- (b) the capsular polysaccharide by means of a specific hydrolysing enzyme,
- (c) the somatic polysaccharides by chemical methods,
- (d) ribonucleic acid by digestion with the enzyme ribonuclease.

The final product, which was free from protein and ribonucleic acid, was able to produce type transformation in a dilution of one in 6×10^8 . The purified product was not affected by proteolytic enzymes, but it was inactivated by a purified preparation of desoxyribonuclease (see also McCarty¹⁴¹). The suggestion is that the principle is a desoxyribonucleotide.

The question as to the chemical nature of the gene which is raised by these observations lies outside the scope of this chapter. One aspect, however, is of interest for us, that of the relation of enzymes and genes. The similarity between enzymes on the one side and genes and gene-like structures on the other has repeatedly been pointed out (Troland¹⁹¹; Muller¹⁵⁶). Genes, like enzymes, are characterized by their catalytic actions and by the specificity of their actions; genes differ from enzymes by their ability of self-reproduction. It is difficult to predict if a distinction based on this difference will prove lasting; too little is known about the mechanism of formation of genes and enzymes to make a classification secure.

Specificity is a feature common to both enzymes and genes, and it has been suggested that the relationship of the gene to its primary 'substrate' is similar to the type of relationship existing between enzyme and substrate⁵⁶. The forces responsible for the specific enzyme-substrate relationship are still very imperfectly known; it seems reasonable to suggest that these forces are similar to those which are responsible for the antibody-antigen reaction. This reaction, which is also characterized by its specificity, has been studied by Pauling and his colleagues, and is ascribed to the action of short-range intermolecular forces¹⁷⁰. Evidence on the connexion between enzyme-substrate and antibody-antigen relationship is scarce, but there are observations which suggest that antibodies acting on certain enzymes compete with the specific substrates of these enzymes. Two enzymes which hydrolyse lecithin have been studied²⁰⁶. One of these enzymes is the lecithinase of snake venom, which hydrolyses one of the ester linkages marked A with the formation of lysolecithin and an unsaturated fatty acid, e.g. oleic acid. The second enzyme, the α -toxin of *Clostridium welchii*, hydrolyses the glycerophosphate linkage of lecithin (at B) with the formation of phosphorylcholine. Antibodies against both these enzymes can be produced; the antisera obtained inactivate the enzymes, each antiserum its specific

enzyme. When lecithin is added to the enzymes prior to the antisera, the degree of inactivation caused by the antiserum is reduced. Experiments are described which suggest that substrate and antibody compete for the enzyme. This indicates that the forces which bring about the attachment of the substrate to the enzyme are similar to those which make the antibody react with the enzyme, its specific antigen.



The lecithin molecule.

1. Ester linkage hydrolysed by cobra venom with formation of lysolecithin.
2. Ester linkage hydrolysed by α -toxin of *Clostridium welchii*.

iv. LOCATION AND EXTRACTION OF ENZYMES

In the study of enzymes, the biochemist usually applies methods which involve the destruction of the structural elements of the cells; this is often unavoidable if it is the aim to obtain highly active and purified preparations. The cytologist, on the other hand, is interested in the localization of the enzymes in the tissues and cells, and in the sites of enzyme formation.

A distinction used to be made between enzymes occurring in the extracellular fluids and secretions on the one hand, and the intracellular enzymes on the other; of the latter, it was long believed that their activity was characteristic of the living cell. Pasteur held that the presence of living yeast cells was an indispensable condition for the occurrence of alcoholic fermentation, and only the discovery by Buchner of fermentation in cell-free extracts showed that there was no general distinction between intra- and extracellular enzymes.

A classification has been used by Willstätter and his colleagues, based on the ease with which enzymes can be eluted from the tissues. Enzymes are considered by Willstätter to be bound to the protein of protoplasm to form so-called 'symplexes'. Some of these symplexes are split easily and the enzyme can then be obtained in solution; such an enzyme is called a 'lyo-enzyme'. Other enzymes are bound more firmly to the insoluble cell proteins; they are called 'desmo-enzymes' (see review by Bamann and Salzer¹¹). Since there is no very sharp distinction between soluble and insoluble enzymes, it seems questionable if the difference between lyo-enzymes and desmo-enzymes is a fundamental one. It seems likely that the attempt to allot to the different structural elements within the cell certain enzyme functions will lead to a more

promising correlation between structure and function. The methods used for obtaining large amounts of the different particulate elements present in cells and some of the results obtained are described in Chapter 1, Part B.

The ambition of the enzyme chemist is to study an enzymic reaction in a system which contains the complete catalytic system and as little else as possible. The system may contain the substrate, the apoenzyme, the coenzyme, and the necessary inorganic co-factors. Such a system serves as a model of the catalytic system in the living cell. In some instances the attempt to reconstitute the complete catalytic system has been successful, but often the enzymic activity is found to be bound up with particulate matter and it has been impossible to study the activity in a homogeneous system. Cytochrome oxidase is an example of an enzyme which has not been isolated. Another example is the enzyme amine oxidase, an enzyme which occurs in all the vertebrates examined as well as in many invertebrates (e.g. in *Sepia*). In the mammalian liver, the activity is associated with the liver granules (Warburg's 'Körnchensuspension'), mitochondria and microsomes,⁸³ but if the granules are destroyed by dispersing agents, e.g. by lysolecithin or by bile salts, amine oxidase activity is not impaired.

The spatial arrangement in the cell must clearly be of great importance when the catalytic system is complex. Ogston and Smithies¹⁶⁸ have recently suggested that the spatial arrangement of the various enzymes determines the relation between oxidation and phosphorylation in the cell. (See also Monné¹⁵³). A simpler case is that studied by Krebs¹¹⁵ in the oxidation of L-amino-acids; Krebs showed that this reaction took place in the presence of slices of mammalian liver or kidney tissue and in concentrated homogenates of tissue; on dilution, the activity decreased more rapidly than in proportion to the degree of dilution. The interpretation given to these experiments was that the reaction catalysed required three components, viz. enzyme, substrate, and an additional factor, and that on dilution the chances for a ternary collision decreased rapidly. An enzyme which catalyses the oxidation of certain L-amino-acids has since been isolated from mammalian kidney¹⁷, but it is not known if this is the complete catalytic system (Krebs¹¹⁶). A different L-amino-acid oxidase occurs in the venom and the tissues of some snakes; this enzyme is highly active in dilute solution²⁰⁷.

The spatial arrangement of enzyme and substrate within the cell regulates the rate of enzymic intracellular reactions. This was first clearly recognized by Claude Bernard when he discovered in the liver both glycogen and the enzyme diastase which hydrolyses glycogen. He found that the liver of the hibernating frog contained much glycogen and little glucose; conversely, the liver of the summer frog contained much glu-

cose but little glycogen. The enzyme was present in summer as well as in winter; he therefore assumed seasonal differences in the spatial arrangement of enzyme and substrate within the cell. It has been suggested that surface-active agents, e.g. bile salts, may modify the accessibility of the enzyme to the substrate¹⁸⁶.

In the bacterial cell a similar spatial segregation of enzyme and substrate is assumed by Dubos⁵³ in the study of 'flash lysis'. Acetone-dried powders can be prepared of pneumococci in which the single cells are still distinct and display their characteristic staining properties. This powder, when re-suspended in water at a temperature of 30–40°C., undergoes flash lysis in a few seconds; when the powder is suspended in boiling water no lysis occurs. The lysis is interpreted as an enzymic process in which enzymes, released by the acetone treatment from their normal segregation within the cell, act on their specific substrates, which are normally held within separate compartments of the cell; if such substrates are important structural elements, their enzymic decomposition will result in cell disintegration. Suspending cells in boiling water kills the enzymes and thus prevents lysis.

V. ENZYMES WITH LYTIC ACTION

There are many enzymes which have a lytic effect; they act by destroying an important structural constituent of the cell. This cytolytic action is a property of many digestive enzymes: by destroying the cell structure they expose the contents of the cell to the digestive process. Snake venom is a typical secretion of this type. Cobra venom contains a powerful lecithinase which catalyses a reaction with a two-fold effect: firstly, it destroys the lecithin which forms part of the membrane of many cells, e.g. the red blood cells, and secondly, it forms lysolecithin, a compound with strong lytic action. The lecithinase of *Clostridium welchii* does not form lysolecithin; its lytic action is due only to the removal of lecithin.

In bacteria, the chemical composition of the structural elements shows a great variety, and lysis is therefore brought about by many different enzymes, some of which act very selectively. An enzyme chiefly characterized by its lysing action is lysozyme, discovered by Fleming in nasal mucus; it also occurs in egg-white. This enzyme lyses a number of bacterial species. Its specific substrate was shown to be an amino-sugar of high molecular weight^{59,149}.

Other enzymes exist which act on extracellular structures. Many bacteria contain fibrinolysins; these have been shown to have enzyme character (MacFarlane and Biggs¹⁴²). These lysins show a high degree of substrate specificity. Another enzyme which determines the invasive character of many bacterial infections was first described as 'spreading factor'; it was found in a number of bacteria and also in the mammalian

testis^{54,143}. Chain and Duthie showed that this factor was an enzyme and that its specific substrate was hyaluronic acid, a polysaccharide which consists of glucuronic acid and N-acetylglucosamine^{36,148}. Hyaluronic acid occurs not only in the connective tissue, but the same substance forms the capsule of streptococci; destruction of the capsular material by hyaluronidase reduces the virulence of the streptococci.

Enzymic hydrolysis of the capsular polysaccharides has been particularly studied in pneumococci; hydrolysing enzymes have been obtained from saprophytic micro-organisms which are highly specific for each pneumococcal type (Dubos⁵³).

For a more detailed discussion of the enzyme character of bacterial toxins the reader is referred to a review by Pappenheimer¹⁶⁹.

vi. RESPIRATION ENZYMES

Paul Ehrlich was the first to use cytological methods in the study of cell oxidation. In his book *Das Sauerstoffbedürfnis des Organismus* (1885) he attempted to obtain information on the intensity of tissue oxidation in various organs by intravital injection of dyes which are reduced to leuco-derivatives. Ehrlich's work—which laid the foundation-stone of the technique of intravital staining—did not lead to an understanding of cellular oxidations; specific affinities of the dyes to the structural elements made it impossible to interpret the results obtained. But of the dyes employed by Ehrlich two have proved of great importance in the study of biological oxidations: methylene blue in the work on dehydrogenases, and indophenol in the work on the cyanide-sensitive oxidation catalysts. Dehydrogenases and 'indophenol' oxidase (now called cytochrome oxidase) are two main types of intracellular oxidation catalysts. They are briefly reviewed in the following, in as far as they are accessible to cytological examination.

The importance of iron in cellular respiration was first recognized by Warburg¹⁹⁵. His discovery that the chief respiratory catalyst (Warburg's respiratory enzyme) contained haemin-iron is not based on direct spectroscopic observation but on more indirect evidence: cellular respiration is inhibited by carbon monoxide, and the inhibition is reversed by light. Dissociation by light is known to be a property of iron-CO compounds. Warburg determined the relative efficiencies of monochromatic light of different wave-lengths in reversing the carbon monoxide inhibition of cell respiration and showed that the 'efficiency spectrum' thus obtained was that of a haemin compound. Since 1925 Keilin has analysed the functions of the intracellular haemin compounds (McMunn's histo- and myohaematin). He examined these substances by direct spectroscopic examination; they were shown to be present in oxidized and reduced form, and he called them 'cytochromes'. They occur in most living cells.

Tissues suitably illuminated show the red colour of cytochrome; this can be seen in a slice of brain tissue carefully washed free of blood, or in a thick suspension of yeast, observed in transmitted light. Suitable materials for the study of the cytochromes in cells are the thoracic muscles of living insects (*Galleria*) or suspensions of baker's yeast. Keilin¹⁰⁶ showed that the typical cytochrome spectrum was due to the

	<i>a</i>		<i>b</i>	<i>c</i>	<i>d</i>	
Cytochrome	α_1		α_2	α_3	β_1	β_2
Compound α'	α_1				β_1	
" <i>b'</i>			α_2		β_2	
" <i>c'</i>				α_3		β_3
	Red				Blue	

TEXT-FIG. 1. The upper line gives the absorption bands at reduced cytochrome. The three lower lines give the position of the bands of the components *a*, *b*, and *c*. After Keilin¹⁰⁶.

610	605	600	595	590	585	580	575	570	565	560	555	550	545	540	535	530	525	520
6032	<i>a</i> <i>Bacillus subtilis</i>							5660	<i>b</i>		5502	<i>c</i>					5210	<i>d</i>
6035	<i>a</i> <i>Yeast cells</i>							5645	<i>b</i>		5490	<i>c</i>					5190	<i>d</i>
6035	<i>a</i> <i>Eschallot bulb</i>							5640	<i>b</i>		5500	<i>c</i>					5190	<i>d</i>
6046	<i>a</i> <i>Bee wing muscles</i>							5665	<i>b</i>		5502	<i>c</i>					5210	<i>d</i>
6038	<i>a</i> <i>Dytiscus wing muscles</i>							5664	<i>b</i>		5495	<i>c</i>					5205	<i>d</i>
6046	<i>a</i> <i>Galleria wing muscles</i>							5657	<i>b</i>		5495	<i>c</i>					5200	<i>d</i>
6035	<i>a</i> <i>Snail radula</i>							5650	<i>b</i>		5495	<i>c</i>					5200	<i>d</i>
6040	<i>a</i> <i>Frog heart muscles</i>							5660	<i>b</i>		5500	<i>c</i>					5205	<i>d</i>
6045	<i>a</i> <i>Guinea-pig heart muscles</i>							5662	<i>b</i>		5500	<i>c</i>					5205	<i>d</i>

TEXT-FIG. 2. Positions of the four absorption bands *a*, *b*, *c*, and *d* of reduced cytochrome in various organisms. After Keilin¹⁰⁶.

presence of at least three substances, which he called cytochromes *a*, *b*, and *c* (see Text-fig. 1). They give typical haemin spectra; the bands of the reduced cytochromes from different sources are shown in Text-fig. 2. A thick suspension of baker's yeast shows the bands of reduced cytochrome which disappear on shaking with air. The cytochrome has been oxidized. If the suspension is left standing, the cytochrome is again reduced. The rate of reduction is dependent upon the presence of substrates for oxidation. Reduction occurs rapidly if the cells are saturated with substrates, and slowly if they are relatively starved. The oxidation

of cytochrome on shaking is not due to an autoxidation: the rate of oxidation is slowed down by cyanide or carbon monoxide, and these substances do not combine with the cytochromes *a*, *b*, or *c*, as there is no change in the spectrum on the addition of CO or KCN. These inhibitors act on an enzyme which oxidizes cytochrome: cytochrome oxidase. Cytochrome oxidase exhibits all the characteristic properties of Warburg's respiratory enzyme. Moreover, he showed that the enzyme was identical with the indophenol oxidase of mammalian tissue. This enzyme catalyses the oxidation of the 'nadi' reagent (dimethyl-*para*-phenylenediamine + α -naphthol) to indophenol and that of *para*-phenylenediamine to a diamine. Keilin and Hartree^{107,108,109} have shown that the enzyme does not directly oxidize these substances: they are oxidized by oxidized cytochrome. The reoxidation of the reduced cytochrome by atmospheric oxygen is catalysed by the cytochrome oxidase. Quite a number of substances are oxidized by the cytochrome—cytochrome-oxidase system in a similar manner, e.g. polyphenols (adrenaline), ascorbic acid, sulphhydryl compounds (cysteine). Cytochromes seem to be the only substances which are directly oxidized by the cytochrome oxidase.

In addition to the three cytochromes *a*, *b*, and *c*, Keilin and Hartree have described a component which they call cytochrome *a*₃. This substance displays remarkable similarities to cytochrome oxidase. Apart from component *c*, the different compounds have not yet been isolated, and their characterization is based on the observation of the absorption spectra. In the following paragraphs a short summary of the properties of the components, as recently described by Keilin and Hartree, is given:

Component *a* is a haem-protein compound, insoluble or intimately bound to the insoluble material of the cells. It is thermolabile and does not combine with CO, KCN, or other inhibitors.

Components *a*₁ and *a*₂ are found only in a few bacteria devoid of component *a*.

Component *a*₃ is thermolabile; it reacts with CO and KCN; it is autoxidizable. It shares these properties with the respiratory enzyme, cytochrome oxidase. It is not certain whether component *a*₃ is in fact identical with cytochrome oxidase, since (1) the CO compound of *a*₃ does not dissociate in light, and (2) the oxidized component *a*₃ does not oxidize anaerobically the reduced component *c*.

Component *b* has also up to now proved insoluble. In contrast to components *a* and *c*, it is somewhat autoxidizable. In the presence of urethane and a suitable substrate, e.g. succinic acid, component *b* remains reduced, whereas components *a*, *a*₃, and *c* are oxidized. There is reason to believe that substances like polyphenols (which react with cytochrome without a specific activator) do not require this component,

but that it is required for the oxidation of activated substrate molecules such as succinic acid (see below).

Component *c* is the only one that is soluble; it has been separated and prepared in a pure condition; it is relatively thermostable. It does not combine with CO or KCN. It is not autoxidizable under physiological conditions.

The positions of the characteristic absorption bands of the different components are given in the accompanying table (after Keilin and Hartree¹⁰⁸).

<i>Components of cytochrome</i>	<i>Notation of bands</i>		<i>Position in mμ</i>
	<i>Previously used</i>	<i>Proposed now</i>	
<i>a</i>	<i>a</i>	<i>aα</i>	605
	..	<i>aβ</i>	?
	..	<i>aγ</i>	452
<i>a₃</i>	..	<i>a₃α</i>	600
	..	<i>a₃β</i>	?
	..	<i>a₃γ</i>	448
<i>b</i>	<i>b</i>	<i>bα</i>	564
	<i>d</i>	<i>bβ</i>	530
	..	<i>bγ</i>	432
<i>c</i>	<i>c</i>	<i>cα</i>	550
	<i>d</i>	<i>cβ</i>	521
	..	<i>cγ</i>	415
<i>a₃·CO</i>	..	<i>a₃COα</i>	590
	..	<i>a₃COβ</i>	?
	..	<i>a₃COγ</i>	432
<i>a₃·CN</i>	..	<i>a₃CNα</i>	590
	..	<i>a₃CNβ</i>	?
	..	<i>a₃CNγ</i>	450

In higher animals the typical cytochrome spectrum as described above is most easily seen in heart and skeletal muscle as well as in the grey matter of the brain. Keilin and Hartree originally assumed the typical cytochrome spectrum to be absent in other organs, but Slater, working in Keilin's laboratory in Cambridge, has recently shown that the cytochromes of mammalian kidney agree in all essentials with those of heart¹⁸⁷.

Most of the normal oxidizable substrates of the tissues differ from substances like paraphenylenediamine or adrenaline in that they are not directly oxidized by the cytochrome—cytochrome oxidase system; they require the presence of a catalyst which has a high degree of substrate specificity. The interrelationships between the cytochrome

two bands at 455 m μ and 495 m μ which might be due to the presence of flavin in the human retina.

Pyridino-proteins. The enzymes of this group contain nicotinic amide (Warburg) as well as adenine. The coenzyme of alcoholic fermentation (Harden and Young) is a prosthetic group of this kind. These substances are colourless, but they have characteristic absorption bands in the ultraviolet range. The pyridino compounds have an absorption band at 260 m μ . The dihydropyridine (dihydro-coenzyme) has a new band at 345 m μ . According to Warburg, the dihydropyridines show a marked whitish fluorescence in ultraviolet light. The pyridines do not show this fluorescence. In sections of living liver tissue small granules can be seen in ultraviolet light which show a brilliant white fluorescence. This fluorescence, which fades very quickly, may well be due to the dihydropyridines present. A great number of specific dehydrogenases contain coenzymes of this group. The pyridino group is reduced to dihydropyridine during the activity of these enzymes. One of the flavo-proteins, diaphorase, acts by reoxidizing the dihydro-*l*-pyridine formed. Nicotinic acid is the pellagra-preventing factor of the vitamin B complex.

Thiamino-proteins. Under the fluorescence microscope in alkaline reaction the green fluorescence of liver tissue due to flavin disappeared and was replaced by a bluish fluorescence. We now know that this fluorescence is due to the presence of thiamine in the tissue. This substance is known to be identical with vitamin B₁, now known as aneurin or thiamine. In combination with phosphoric acid it is a component of those enzymic systems which metabolize pyruvic acid. The oxidative metabolism of pyruvic acid in the animal in relation to thiamine was first studied by Peters and his collaborators in Oxford; the mechanism of the reaction is here more complex than in yeast, where diphosphothiamine acts as co-carboxylase in the reaction¹³⁹:



An increase in the amount of extractable thiamine in stimulated nerve has been reported¹⁵⁸.

vii. OTHER ENZYMES CONTAINING HEAVY METALS

a. Iron. The cytochrome system is not the only enzyme which contains haemin-iron. Two other systems may be briefly mentioned:

(α) *Catalase*: it catalyses the reaction:

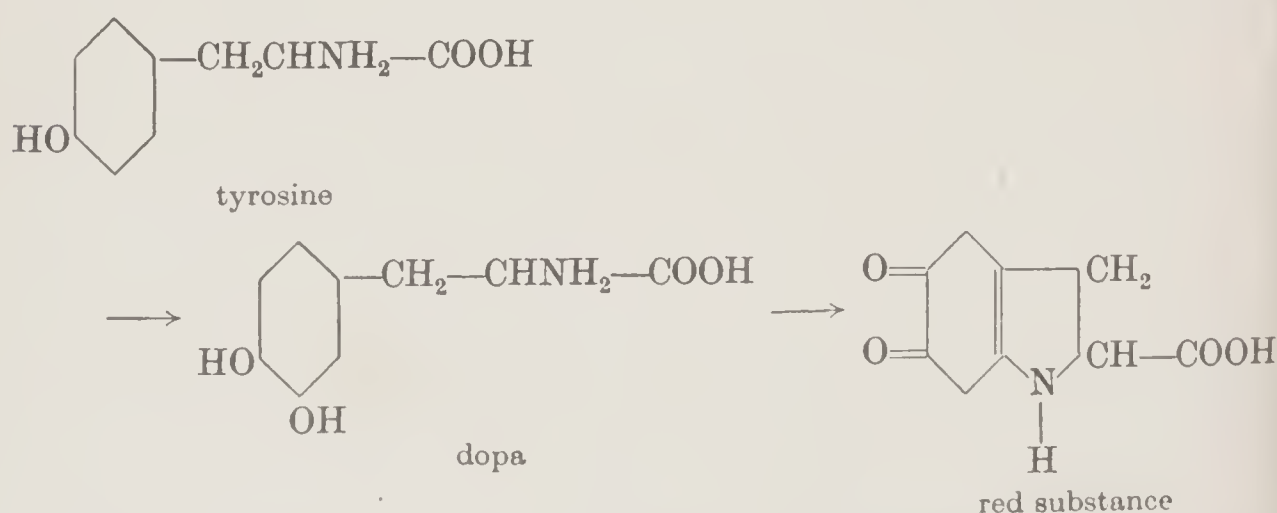


This reaction, which has been known for a long time, may be called the 'catalase' action of the enzyme; the importance of the enzyme for cellular metabolism may be due to its 'peroxidase' reaction: the oxidation of alcohol to acetaldehyde in the presence of 'nascent' peroxide.

The enzyme is almost ubiquitous; only a few anaerobic bacteria are free of catalase.

(β) *Peroxidase*: it catalyses the oxidation of a number of oxidizable substrates in the presence of peroxide. It is chiefly found in plants; horse-radish usually serves as a source of the enzyme. Peroxidase has a typical absorption spectrum with bands at 645, 583, 458, and 498 $m\mu$. Keilin and Mann¹¹⁰ have seen the band at 645 $m\mu$ when they examined a slice of horse-radish root of 1 cm. thickness with a microspectroscope. In the plant peroxidases, the iron is part of a protohaem group. In mammals, peroxidases occur in the mammary gland (lactoperoxidase) and in the granula of polymorph leucocytes (myeloperoxidase). The latter enzyme is responsible for the peroxidase reaction of the leucocytes, the formation of a coloured compound from benzidine, and similar substances, in the presence of hydrogen peroxide. The mammalian peroxidases differ from the plant enzymes: they contain a green haem compound different from protohaem; they are therefore called verdo-peroxidases^{4,205}. Possibly they are chemically closely related to cytochrome oxidase.

b. Copper is present in a number of enzymes which oxidize phenols (monophenoloxidase, polyphenoloxidase, laccase) and in the ascorbic acid oxidase of plants. Monophenoloxidase (or tyrosinase) differs from the polyphenoloxidase in that it attacks monophenols, e.g. tyrosine; it occurs in many plants (e.g. potatoes or mushrooms) and in insects (e.g. the meal-worm); it also occurs in the ink gland of *Sepia officinalis*. The reaction catalysed by the enzyme has been cleared up by the work of Raper; tyrosine is oxidized to 3:4-dihydroxyphenylalanine (usually briefly called dopa), and this substance is further oxidized to a red precursor of melanin:



c. Dopa oxidase. In 1917 the Swiss dermatologist Bloch²⁰ described a reaction of the mammalian skin. He treated unfixed frozen sections (in agar) with dilute solutions of dopa. There appeared after some time a dark pigmentation in many cells of the basal layer of the epidermis.

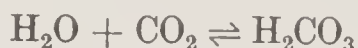
and especially in the so-called dendritic cells—cells which usually show pigmentation. This reaction was not found in albinotic skin; it was intensified in skin previously irradiated. Bloch believed that the reaction was strictly specific and characteristic of the cells which formed pigment (melanoblasts), and he distinguished them from the cells of the dermis which only stored pigment and which did not give the dopa reaction (melanophores). Many investigators now believe that pigment formation in the skin only occurs in the dendritic cells. A simple technique of demonstrating the dopa oxidase reaction in paraffin sections has been described by Becker¹⁴.

The dopa reaction in the embryonic eye has been studied by Miescher¹⁵⁰. In the chick embryo a positive reaction begins to appear at the end of the third day in the pigment epithelium of the retina, just about at the time when the pigment formation begins. The reaction becomes more intense in later stages, reaches a maximum, and decreases until it is negative again on the 18th–20th day. In the pigment cells of the choroid, the ciliary body, iris, and pecten the reaction also is first seen when pigmentation occurs and becomes negative after the pigment has been fully formed. Similar changes take place in the mammalian eye.

The theory advanced by Bloch and his collaborators was that a precursor of melanin circulated in the blood and that from it pigment was formed by the specific enzyme present in the melanoblasts. Bloch believed that dopa was this precursor, and the dopa oxidase the specific enzyme. He claimed that *l*-dopa was the only substrate of the enzyme. This is to-day no longer maintained; a number of other phenols (hydroxytyramine (Mulzer and Schmalfluss¹⁵⁷), *p*-hydroxyphenylpyruvic acid, and 3:4-dihydroxyphenylpyruvic acid (Moncorps¹⁵²) give a similar reaction. In the rabbit, epinine (3:4-dihydroxyphenylethylmethylamine) has been found to give the dopa oxidase reaction even better than dopa itself; based on this observation, Danneel⁴² suggests that epinine is, in fact, the precursor of melanin. In this connexion it must be mentioned that phenoloxidases have so far not been shown to be present in mammals. Moreover, it has already been mentioned that the cytochrome system also oxidizes polyphenols.

As to the chemical nature of the enzyme system involved, there is little known. Extracts from melanotic tumours of the mouse skin oxidize tyrosine and 3:4-dihydroxyphenylalanine^{82,88}, just as preparations from *Sepia*. This might suggest that the enzyme is related to the copper-containing enzymes of invertebrates. This might fit in with reports of an interference with normal pigment formation due to the administration of phenylthiocarbamide, a substance which forms complexes with copper¹⁷⁵. On the other hand, Greenstein reports that in the presence of *p*-phenylenediamine and cytochrome *c*, extracts from melanotic tumours show an increased oxygen uptake (see also Burk *et al.*³³).

d. *Zinc. Carbonic Anhydrase.* This enzyme was first discovered by Meldrum and Roughton; it catalyses the reaction



in both directions. The enzyme has been shown by Keilin and Mann^{111,112} to be a zinc-protein compound. The importance of the enzyme for CO₂ transport in mammalian blood has been fully discussed by Roughton¹⁸²; the enzyme is found in the red corpuscles only; in fact, in some species the plasma contains an inhibitor for the enzyme (Booth²⁶).

Keilin and Mann¹¹² have described a method by which the role of the enzyme in the transport of carbon dioxide in the red corpuscles can be made visible.

In the mammal, carbonic anhydrase is also found in the gastric mucosa (Davenport and Fisher⁴³). Keilin and Mann have obtained a preparation of the enzyme from pig's stomach which was probably about 30 per cent. pure; they showed that not more than 0.75 per cent. of its activity could have been present in the red blood corpuscles of the mucosa. Davenport found that the fundic region of the mucosa had a much higher activity than the pyloric region. As the oxyntic cells of the stomach occur in the fundus only, the correlation between enzymic activity and distribution of oxyntic cells was studied. Davenport adapted the method of Linderstrom-Lang (see below) for the determination of carbonic anhydrase. The gastric mucosa of rats, dogs, and cats were used. From his findings Davenport concluded that the enzyme was localized in the oxyntic cells and that they contained 5–6 times as much enzyme as the red blood corpuscles. The peptic cells of the gastric glands were found not to contain the enzyme; the cells of the gastric surface epithelium had about one-tenth of the activity of the oxyntic cells.

The exact nature of the importance of carbonic anhydrase for the secretion of hydrochloric acid by the gastric mucosa remains to be cleared up. In Davenport's experiments, the carbonic anhydrase of the mucosa was inhibited by sodium thiocyanate (NaCNS); this also suppressed gastric secretion. Feldberg, Keilin, and Mann⁶¹ confirmed this result, but in their experiments sulphanilamide, a much more potent and specific inhibitor of the enzyme, failed to inhibit the secretion of hydrochloric acid in the cat, in concentrations which strongly inhibited carbonic anhydrase activity *in vitro*. These experiments left the question unanswered, why the oxyntic cells should contain such a high enzymic activity.

Recently, methods have been worked out by Davies⁴⁴ for the study of acid secretion in the isolated gastric mucosa of frogs and toads. In this method the mucous membrane is dissected off the muscular coat of the stomach, and the mucosa is tied, with the lumen inward, to form a sac; in such a preparation the acid secreted is not neutralized by the

alkali liberated at the same time on the basal surface of the preparation. Stimulation of acid secretion by histamine can be observed. Using this method, Davies and Edelman⁴⁵ have found an inhibition of both acid secretion and carbonic anhydrase activity by *p*-toluene sulphanilamide in a concentration of $1.7 \cdot 10^{-2}$ M. The authors explain the negative results of Feldberg *et al.* by the incomplete inhibition of carbonic anhydrase in the living animal. According to Davies, the enzyme is necessary to ensure rapid formation of bicarbonate ions from CO_2 and OH^- ions formed in the reaction:



Observations of the occurrence of carbonic anhydrase in teleost fishes are of interest. In 1932 Keys and Willmer¹¹³ described in the gills of the eel (*Anguilla vulgaris*) and other teleosts eosinophil cells similar in appearance to the oxyntic cells of the gastric mucosa. These cells are secretory cells; they are situated in the proximal parts of the gill leaflets, especially on the side nearest to the gill rays (see Plate I, Fig. 1). They are therefore in close contact with the venous blood entering the gills. Keys and Willmer ascribe to these cells, especially in marine species, the function of excreting chloride from the blood into the sea-water. Leiner^{122,123} describes these cells again and finds them not only in the gills but many more of them in the pseudobranchs, a preponderantly glandular structure situated on the roof of the palate. The organ contains a vascular network which gives its blood to the major ophthalmic artery. Leiner finds a very high carbonic anhydrase activity in the pseudobranchs; the gills also contain the enzyme. He believes that the enzyme occurs in the eosinophil cells, and that they secrete the enzyme into the blood. However that may be, it is interesting that these two types of cells of similar appearance, the oxyntic cells of the gastric mucosa and the eosinophil cells in teleosts, should contain carbonic anhydrase in high concentration. Carbonic anhydrase is also found in the teleostian eye (choroid, choroid body, retina).

In the invertebrates, carbonic anhydrase occurs also in the gills (lobster, squid). *Limulus* contains the enzyme in the white cells. (Robertson and Ferguson¹⁷⁷; Ferguson, Lewis, and Smith⁶⁷; van Goor⁸¹.)

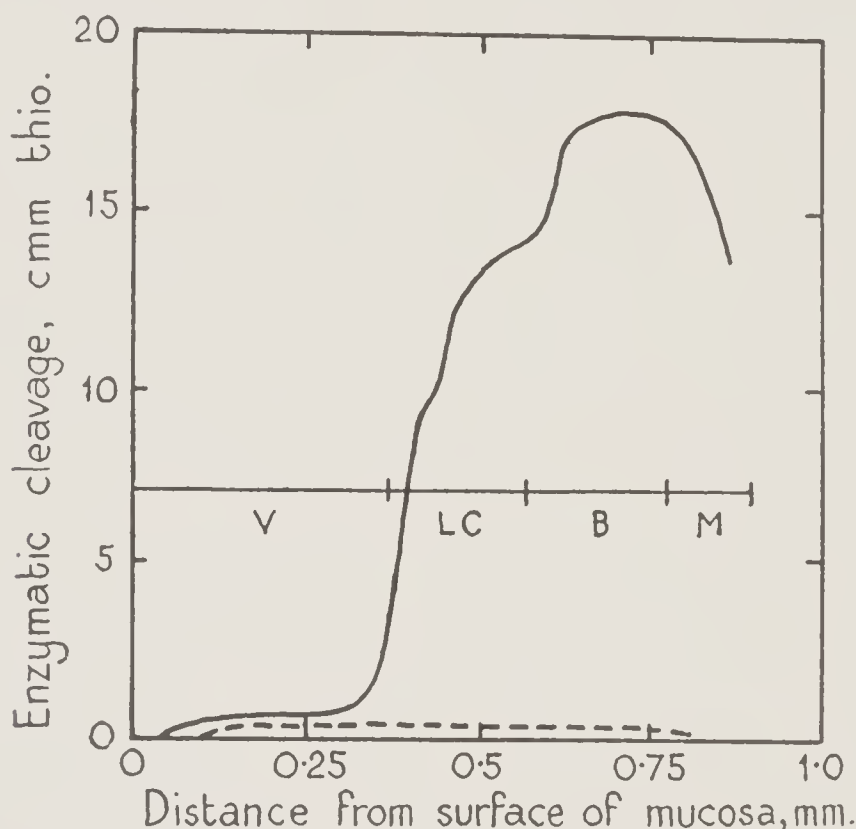
viii. HYDROLYSING ENZYMES

Linderstrøm-Lang¹²⁴⁻¹³⁶ and his collaborators in Copenhagen have worked out micro-methods for the determination of a number of enzymes in microtome sections. The fresh tissue is frozen and a cylinder of small diameter stamped out and cut on a freezing microtome; alternate sections are used for the enzyme analysis and for the histological control.

a. Carbohydrate-splitting Enzymes

Amylase. In this way Linderström-Lang and Engel¹²⁸ analysed the distribution of amylase in the barley grain. The cells bordering the aleuron and starch cells showed the highest enzyme content, about 20 per cent. of the total amylase content of the grain.

Van Genderen and Engel⁷⁵ also studied the distribution of this enzyme in the mucous membrane of the gut. Horizontal sections were made through the wall of the gut, parallel to the free surface; the first few

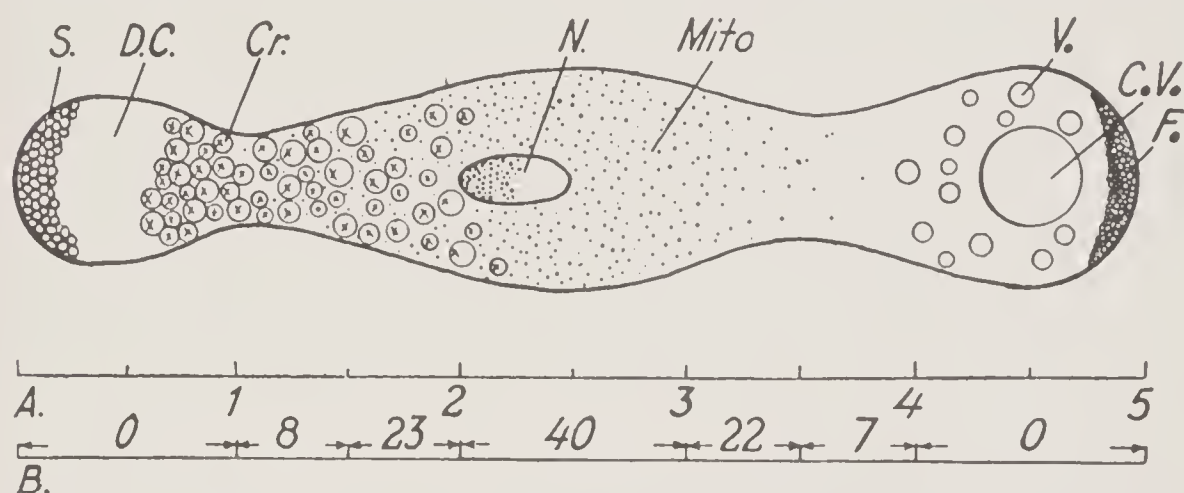


TEXT-FIG. 3. Amylase activity in the duodenal mucosa of the rabbit. Ordinates: c.mm. N/20 $\text{Na}_2\text{S}_2\text{O}_3$. Abscissae: V = level of villi; LC = level of crypts of Lieberkühn; B = level of Brunner's glands; M = level of muscle coat. After van Genderen and Engel⁷⁵.
— amylase activity; ---- blanks.

sections through the block of tissue contained the villi only; later sections went through the region of the crypts of Lieberkühn, still deeper sections through the muscularis mucosae, while the last sections went through the submucosa, which in the case of the duodenum contains Brunner's glands. Enzymic activity was measured by incubating a 25μ section of a diameter of 2.36 mm. for 40 minutes at 32°C . with a standard amount of starch. At the end of the experiment the amount of starch left over was determined iodimetrically. The curve in Text-fig. 3 shows the amount of N/20 $\text{Na}_2\text{S}_2\text{O}_3$ used for the free iodine. Thus the curve expresses directly the amylase activity in the sections. It was found that in the rabbit the sections through Brunner's glands in the submucosa contained the largest amount of amylase. Brunner's glands of the rabbit contain serous and mucous cells, while those of the rat contain mucous cells only; in the latter animal no amylase was found at

the level of the glands. In the ileum of the rat amylase was found only in the region of the villi, smaller amounts in fasting animals, more after feeding. It is very likely that the enzyme is only adsorbed to the surface of the epithelial cells covering the villi, but not produced by them.

In the case of *Amoeba proteus* Holter and Doyle⁹² were able to investigate the intracellular distribution of amylase. Amoebae were centrifuged so that the cell contents became stratified, and the enzyme



TEXT-FIG. 4. Diagram of centrifuged amoeba. The first scale shows the division in transverse zones; the second scale the percentage of mitochondria found in each zone. For abbreviations see text. After Holter and Doyle⁹².

content was then determined in sections made parallel to the plane of stratification. Text-fig. 4 shows the stratification obtained: fat granules (F.) and vacuoles (V. and C.V.) accumulate near the centripetal pole; dense cytoplasm (D.C.), crystalline (Cr.) and other cytoplasmic inclusions (S.) near the centrifugal pole. The nucleus retains a central position, and a broad middle zone is filled with mitochondria (Mito). It is this region which was found to contain most of the amylase. The enzyme content in each section was proportional to its content of mitochondria; e.g. zones 1 to 3 contained 71 per cent. of the total amylase, and zones 1 to 4 all the amylase of the cell. It was suggested that the mitochondria are the carriers of this enzyme. This is of interest in view of the probable association of other enzymes with these structures in liver cells.

Maltase. Van Genderen and Engel⁷⁵ also investigated the distribution of maltase in the ileum of the rat. In fasting animals they found most of the enzyme in sections through the villi and the upper parts of the crypts of Lieberkühn, but in the ileum of a rat killed 50 minutes after feeding the crypts of Lieberkühn did not contain this enzyme. If the enzyme were formed in the intestinal mucosa one would expect to find at least the same amount after feeding. These results suggest another explanation: that the enzyme is of pancreatic origin and is only adsorbed on the epithelial cells of the villi and upper part of the crypts. On the other hand, it is interesting to note that in fishes which have no crypts of Lieberkühn, maltase has not been found in the intestinal mucosa¹⁹⁴.

b. Lipolytic enzymes

Lipase. Glick⁷⁶ has investigated the distribution of this enzyme in the mucosa of the pig's stomach and duodenum, using Linderström-Lang's technique. Lipase was found to occur in approximately equal concentrations in the mucous membrane of the cardia, fundus, pylorus, and duodenum. The prismatic cells lining the surface, and the gastric pits in the three parts of the stomach, as well as the glands in the cardiac and pyloric region, contained the enzyme, while the glands of the fundus showed a slightly smaller activity. All parts of the duodenal mucosa contained a fairly high concentration of lipase, the greatest activity being found in the villi of the duodenum.

The results obtained by Jeker¹⁰¹, who made a histochemical investigation of fat resorption in the small intestine of rats, are of interest in this connexion. Two methods were used on frozen sections: (1) Sudan III, which stains neutral fats well; (2) Fischler's method⁷⁰, which was claimed by its originator to show the presence of fatty acids, especially oleic acid. Although the latter method, which is based on the ability of fatty acids, when treated with cupric acetate, to form copper soaps which can then form black lakes with haematoxylin, has since been shown to be relatively unspecific, Jeker's results are nevertheless indicative of the action of lipase within intestinal cells. The epithelial cells covering the villi show a considerable content of black granules when treated by Fischler's method 30 minutes after feeding 3 c.c. olive oil; very little fat can be shown with Sudan III at this stage. A few hours later the reverse is observed; the majority of droplets observed are intensely sudanophil, while only traces of material staining positively by Fischler's method can be found. These changes are illustrated in Plate I, Figures 2, 3, 4, and 5.

In the case of liver cells, Behrens¹⁵ was able to separate the nuclei from the cytoplasm by centrifugal methods. He found the lipase content of the cytoplasmic fraction 20 times higher than that of the nuclei. The small amount of lipase in the nuclear fraction was probably due to contamination with cytoplasmic material.

Recently Gomori⁸⁰ has published a method for the histochemical demonstration of lipase activity. This method depends on the principle that when acetone-fixed tissues containing the enzyme are incubated at pH 6.8–7.4 with a substrate consisting of certain water-soluble commercial esters of fatty acids (Tween 40, Tween 80, or Product 81) in the presence of calcium ions, the fatty-acid radicals split off by the action of the enzyme become trapped and precipitated at the site of enzyme activity. By these means it is claimed that lipase activity is associated with cytoplasmic granules. The conditions which must be satisfied before a technique of this kind can be regarded as demonstrating the cytological localization of an enzyme have been discussed in

Chapter I, Part B. The lipase technique has not yet been investigated from this point of view, and until this has been done its results must be interpreted with caution.

c. Proteolytic enzymes

Pepsin. The distribution of this enzyme in the mucosa of the pig's stomach was investigated by Holter and Linderstrøm-Lang⁹⁶, using horizontal sections through the mucous membrane. They used edestin (2 per cent.) as a substrate, and measured the amino groups set free by the enzyme after 2 hours at 40° C. (pH 2.1) by titrating with N/20 HCl in acetone; 1 c.mm. N/20 HCl corresponds to 0.7 μ g amino nitrogen¹²⁴. The enzyme activity can be expressed as units, defined as the amount of pepsin which splits 0.63×10^{-4} millequivalent peptide linkages of 2 per cent. edestin (pH 2.1) in 14 c.mm. during 2 hours at 40° C.

Pepsin units in a single cell in stomach and duodenum

<i>Cardia</i>	<i>Fundus</i>	<i>Pylorus</i>	<i>Duodenum</i>
Very low in all layers	Surface epithelium and gastric pits 0.2	Surface epithelium and gastric pits 0.07-0.11	Epithelium of villi and crypts 0
	Cell of the neck of the glands 0.2-1.2	Cell of the neck of the glands 0.09-0.16	
	Peptic cell 1.7-2.1	Pyloric gland cell 0.14-0.23	Brunner's gland cell 0.04
	Oxyntic cell 0		

A very high activity was found in sections throughout the fundus, especially those which pass through the deeper part of the tubular glands, where most of the peptic cells are found. The enzymic activity here corresponds to 32-40 units. By contrast, the pyloric mucosa shows a much smaller activity; pepsin was found, especially in the deeper sections through the glands, and amounted to just over 4 units¹³⁴. The pepsin curves in Text-fig. 6 give the distribution of the relative enzyme activities in the three parts of the stomach. It will be noted that a proteinase (pepsin?) which splits edestin at pH 2.1 was also found in Brunner's glands in the duodenum; it occurs there in concentrations similar to those in the cardiac glands. Pepsin is an enzyme which has been prepared in the crystalline state. It can therefore be calculated that a single peptic cell in a gland of the fundus contains about 8 per cent. of its weight (c. 3×10^{-6} mg.) of pepsin. If all pyloric gland cells were engaged in pepsin production the pepsin concentration in each single glandular cell would be about one-tenth of the fundus value.

Production of hydrochloric acid. Heidenhain⁸⁴ suggested that the oxyntic (parietal) cells in the glands of the fundus produce hydrochloric acid and that the smaller, 'chief', or peptic cells produce pepsin. Langley *et al.*¹¹⁸⁻¹²⁰ brought further evidence in favour of this suggestion. They investigated the frog's stomach, where, to a certain degree, glands producing pepsin and hydrochloric acid are separated from each other. Later, Linderstrøm-Lang and Holter^{132,133} made a detailed quantitative study of the acid content of horizontal sections through the mucous membrane of the pig's stomach, which is rather similar to that of man. Most of the oxyntic cells are found in the upper part of the gland of the fundus to a depth of c. 1 mm., while the peptic cells are mainly found in the lower part of these glands. The maximum of acid corresponds to the level of highest frequency of oxyntic cells. The curves for acid and pepsin content are quite different from each other, and correspond very well to the distribution of the two types of cell¹³⁶.

The acid in a 50μ section of 2.36 mm. diameter is neutralized by 2.5 c.mm. M/100 borate. This amount of acid, if distributed evenly through an oxyntic cell, would amount to a concentration of 0.15 N; it is probable that such an enormous concentration can only be present as bound acid. Little is known of the mechanism which leads to the liberation of the acid, but the following two points seem to be of particular interest in this connexion: (1) the high content of carbonic anhydrase in the glands of the fundus, most probably in the oxyntic cells, and (2) the work of Palmer, who showed that there was a maximum of chloride content associated with the maximum of oxyntic cells¹³⁶.

Digestion of keratin. In most animals keratin is not attacked by proteolytic enzymes. The larvae of the clothes moth (*Tineola biselliella* Humm) feed chiefly on keratin. The digestion of keratin in these animals was studied by Linderstrøm-Lang and Duspiva¹²⁷ and Duspiva⁵⁵. The larvae of the wax moth (*Galleria mellonella*), which does not digest keratin, were used for comparison. The surprising observation was made that the proteinase of the intestine of *Tineola* does not digest keratin *in vitro* at the physiological pH (9.6-10.2), though it digests casein actively. The question arose as to how the epithelial cells of the intestine were enabled to digest keratin. This was cleared up by the following experiments. The larvae were fed with wool which had been soaked in various oxidation-reduction indicators. Indigo-disulphonate stained the epithelium and contents of the fore- and hind-gut blue, but in the mid-gut it was partly reduced to a pale green ($E_0 = -0.22$ volts); the fully reduced indicator is yellow. When cotton fibres soaked in indicators were fed the results were even clearer, as cotton is not attacked by the enzyme. In this case blue gallophenine was reduced by the cells of the mid-gut, corresponding to $E_0 = -0.29$ volts. Brilliant alizarin blue ($E_0 = -0.34$ volts), on the other hand, showed no colour change

throughout the intestine. From these observations it can be concluded that the epithelial cells of the mid-gut of the larva of the clothes moth can maintain a reduction potential as low as -0.3 volts.

It is not clear whether this potential is due to the presence of SH groups only or whether other reducing agents (hydrogen carriers) are involved. The important effect of this reduction potential is that the S—S linkages characteristic of the keratin molecule are reduced to —SH groups. The S—S groups form a cross-link between the single peptide chains of the protein and are responsible for the insolubility of keratin. The reduction, therefore, enables the proteinase to attack the keratin. Titration of the free amino and carboxyl groups shows that the proteinase of the intestine of the *Tineola* larva splits peptide linkages. It differs from other proteinases, e.g. that of the pancreas or that found in the wax moth, in that it is not inhibited by free —SH groups.

Peptidases. Peptidases in cells may have different functions, and consequently their localization varies considerably:

Endoenzymes, i.e. peptidases which occur in actively growing cells, where they play their part in building up proteins. In this case they are always diffusely distributed in the cytoplasm, e.g. in unfertilized or fertilized ova, or growing roots of plants.

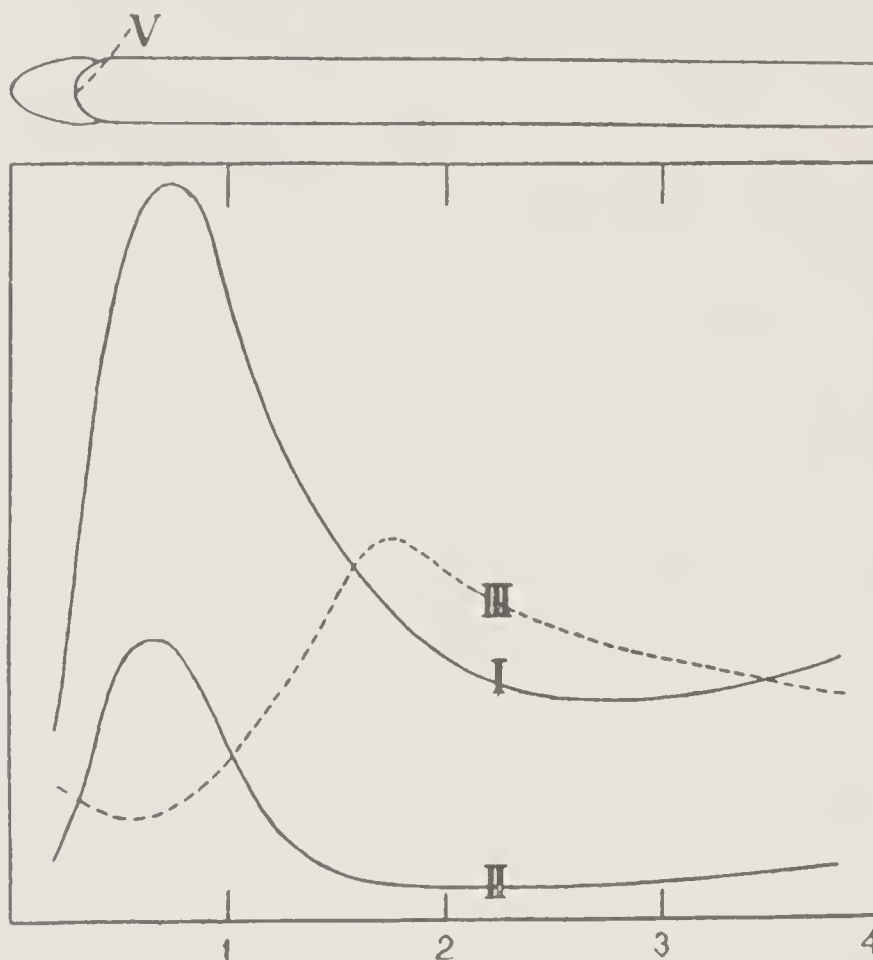
Exoenzymes, i.e. those peptidases which are found attached to specific cell granules, e.g. in the pancreas or intestine, where their role may be either to be excreted or to synthesize specific proteins, e.g. other enzymes.

(a) *Peptidases in growing cells.* The dipeptidases of the ova of marine invertebrates are endoenzymes. They will not act on dipeptides in the surrounding medium unless the cell is killed and the enzyme allowed to diffuse into the medium, where it comes into contact with the substrate. The results quoted below refer to the alanyl-glycine dipeptidase, unless otherwise stated. The unfertilized egg of *Dendraster excentricus* weighing $0.50\ \mu\text{g}$ is able to split $1\ \mu\text{g}$ alanyl-glycine in 1 hour at 40°C .¹³¹ If the egg is cut into two halves so that one contains the nucleus, both parts show an approximately equal dipeptidase activity. From this experiment it may be concluded that at least most of the dipeptidase is in the cytoplasm. This conclusion is supported by the observation that amoebae can be enucleated without effect on their dipeptidase activity⁹³.

The localization of dipeptidase in the cytoplasm was investigated by Philipson¹⁷². He centrifuged unfertilized eggs of the sea-urchin *Psammechinus miliaris*, which were suspended at a boundary between seawater and a sugar solution of a higher specific weight. Under these conditions the eggs separated into a small heavier and a large lighter part. Though the volumes were in the ratio 1:4 both parts had an equal dipeptidase activity. This indicates that the enzyme molecules are

associated mainly with the denser part of the cytoplasm, although it is not possible to connect them with any specific cytoplasmic structure. Similar results have been obtained for the eggs of *Arbacia*, *Echinarachnius*, and *Chaetopterus*^{90,91}.

That fertilization does not lead to an increase of dipeptidase activity in either *Urechis caupo*¹³¹ or *Psammechinus*¹³¹ suggests that in these echinoderms the head of the sperm does not contain dipeptidase.



TEXT-FIG. 5. Distribution of I, alanyl-glycine dipeptidase, and II, leucyl-glycine dipeptidase, in the radicle of the barley germ. Ordinates: enzyme activity; abscissae: distance from tip of the root in mm. Curve III shows the ratio of both enzyme activities. After Linderström-Lang¹²⁴.

V = zone of maximum cell division.

Fifteen minutes after fertilization, however, there is a slight drop in dipeptidase activity, the cause of which is obscure. Three hours after fertilization, i.e. in the 16-cell stage, the original value has been reached, but later, 20–30 hours after fertilization, when there is a decreasing growth rate, the total enzyme activity drops again to half the original value.

No clear correlation appears to exist between enzyme activity and cell determination. In the 8-cell stage of *Psammechinus miliaris* the 4 upper cells are already determined to ectoderm cells and the 4 lower ones will give rise to endodermal and mesodermal structures. However, at this stage no significant difference in alanyl-glycine dipeptidase activity can be found between the blastomeres⁹⁴.

Holter, Lehmann, and Linderstrøm-Lang⁹⁵ investigated a second leucyl-glycine dipeptidase in the case of the eggs of *Tubifex rivulorum*. This enzyme is activated by Mg ions, which was not observed with the alanyl-glycine dipeptidase.

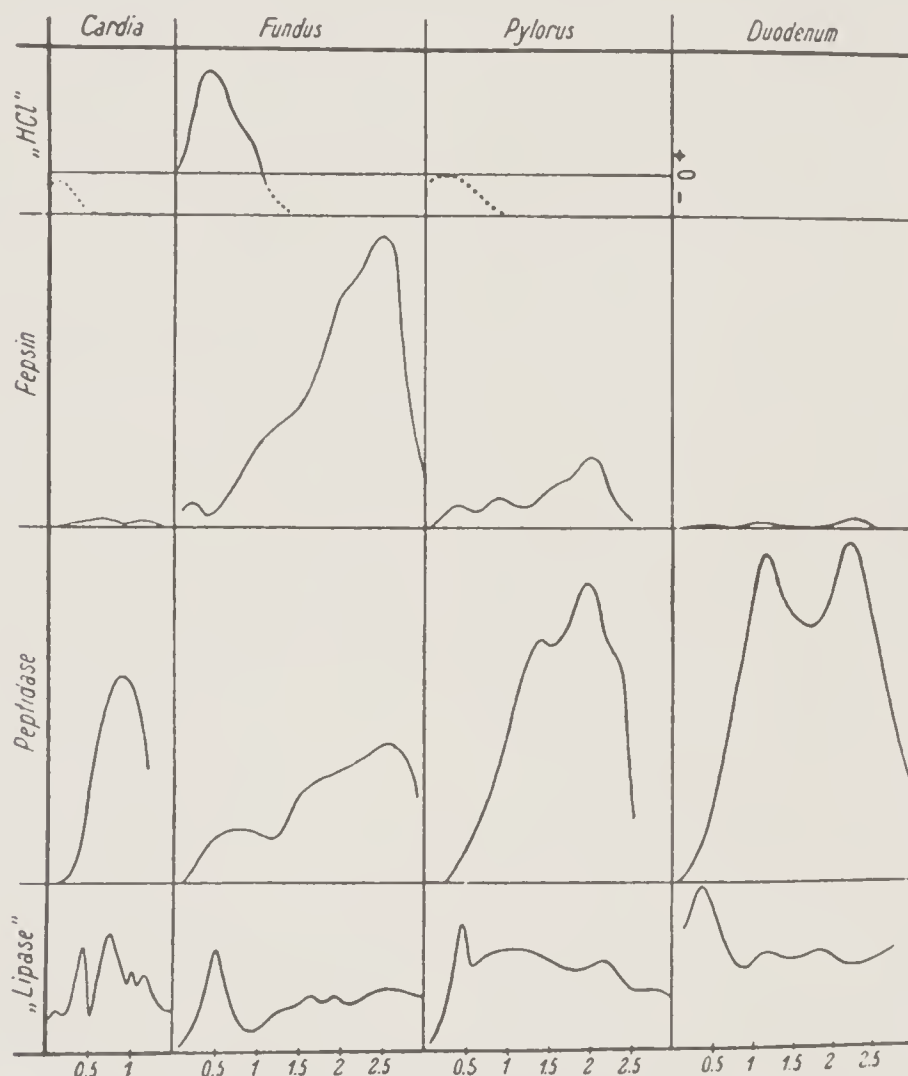
In the radicle of the barley germ (Text-fig. 5) these two peptidases occur in different concentrations at different distances from the tip of the root, the maximum concentration for both enzymes being found at a distance of 0.80 mm. from the tip of the root, i.e. not at the point of maximum cell division (V) but in a region of very active longitudinal cell growth; mitotic divisions were found mainly in the first $\frac{1}{2}$ mm.¹³⁰ The ratio of alanyl-glycine to leucyl-glycine dipeptidase changes in favour of the former with the distance from the tip of the root (see curve III, Text-fig. 5). This suggests that the cytoplasm of the older (longer) cells contains more alanyl-glycine groups than that of the less differentiated younger cells. On the other hand, in the leaf germs both enzymes are evenly distributed, and here zones of cell division and cell growth cannot be distinguished.

(b) *Peptidases of stomach, intestine, and pancreas.* (i) *Stomach.* Alanyl-glycine dipeptidase is not found in the gastric juice but in the mucous membrane of the three parts of the stomach. It is found in the duodenal juice and in the duodenal mucosa. Linderstrøm-Lang and Holter^{132,133} investigated the distribution of the alanyl-glycine dipeptidase in the mucosa of stomach and duodenum of the pig, and details of its distribution in cardia, fundus, and pylorus can be seen in Text-fig. 6. In the mucosa of the fundus the distribution of the enzyme corresponds to that of the peptic cells. The activity of the enzyme was expressed in units which are defined as follows: one unit is the amount of enzyme which splits 0.5×10^{-4} millequivalent peptide linkages in 14 c.mm. 0.1 M alanyl-glycine solution at pH 7.4 in 20 minutes at 30° C.; the short incubation time was used as the peptidase content in the 25 μ sections was considerable. In one section the following values were found: cardia, maximum of nearly 4 units in the glands; fundus, maximum of about 3 units in the region of the peptic cells of the glands; pylorus, maximum of over 5 units in the region of the glands; in the duodenum, maxima of over 6 units, both in Brunner's glands and in the crypts of Lieberkühn.

The deeper levels of the glands of the fundus, where most of the peptic cells are found, show a peptidase activity 10–20 times as high as the pepsin activity. A single peptic cell of the glands of the fundus is able to split 4.5×10^{-7} mg. alanyl-glycine in 20 minutes at 30° C., i.e. 0.15 times its own weight¹³⁶. This activity is about half that of the rapidly growing egg of *Dendraster*, which splits 0.3 times its own weight in similar conditions. Similarly, a single pyloric gland cell hydrolyses 0.68 times its own weight of alanyl-glycine, i.e. the dipeptidase activity is

4.5 times greater than in the peptic cells of the fundus glands. On the other hand, it will be remembered that the cells of the pyloric gland cells contain 10 times less pepsin than the cells of the glands of the fundus. There does not seem to be any clear correlation between these two enzymes.

A dipeptidase for glycyl-*l*-proline was found in the pig's stomach;



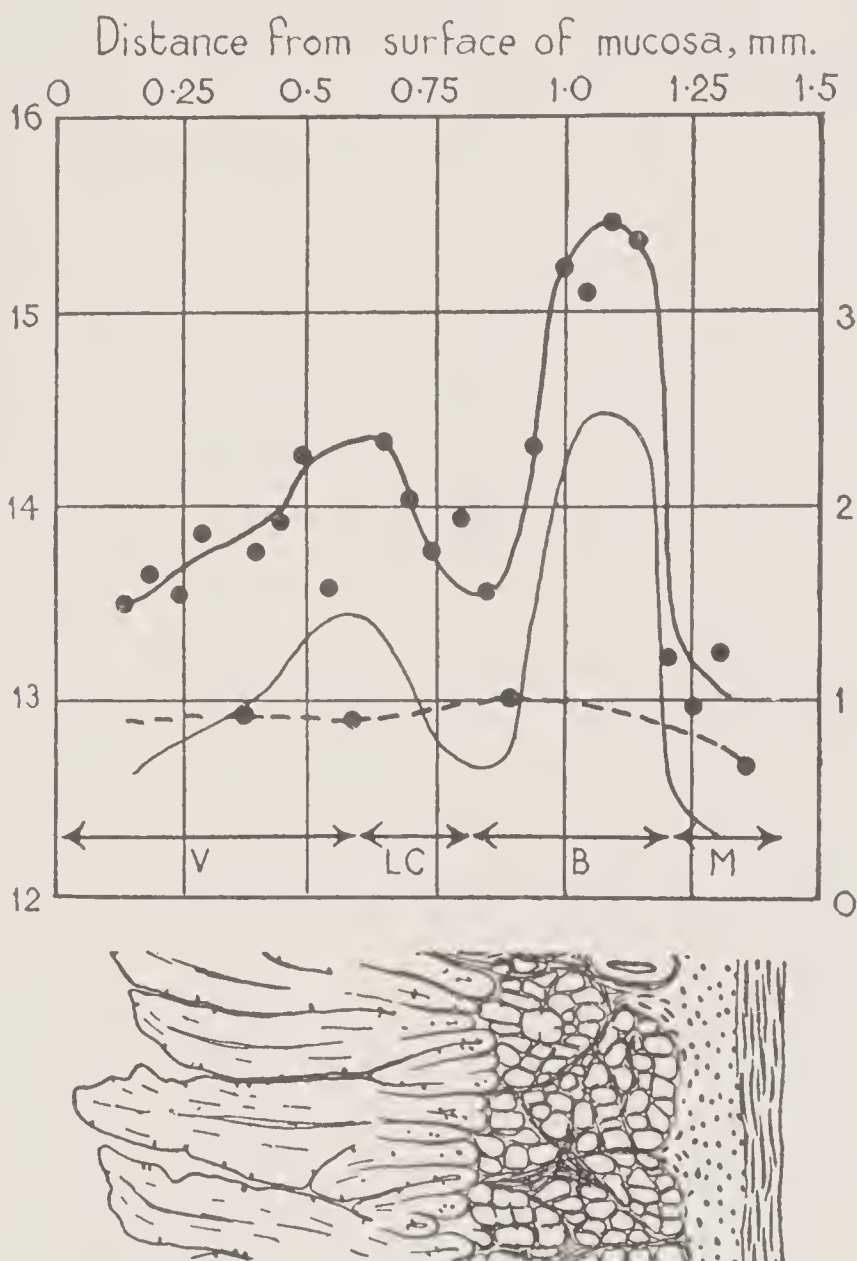
TEXT-FIG. 6. Diagram of relative enzyme activities (ordinates) of the mucosa of the pig's stomach. Abscissae: distance in mm. from the surface. For details see text. After Linderström-Lang¹²⁵.

this enzyme was present in all layers of the fundic and pyloric mucosa. No carboxypolypeptidase is found in the stomach, but a fairly active aminopolypeptidase which occurs in fundus and pylorus in a distribution similar to that of the alanyl-glycine polypeptidase.

(ii) *Intestine*. There are two maxima in the distribution of alanyl-glycine dipeptidase activity in the mucous membrane of the pig's duodenum: one coincides with the crypts of Lieberkühn, the other with Brunner's glands. In both the alanyl-glycine dipeptidase activity of 25 μ sections amounted to 4–6 units as defined above.

Similar results with glycyl-glycine as substrate were obtained in the case of the duodenum of the rat⁷⁵ (Text-fig. 7). The distribution of this

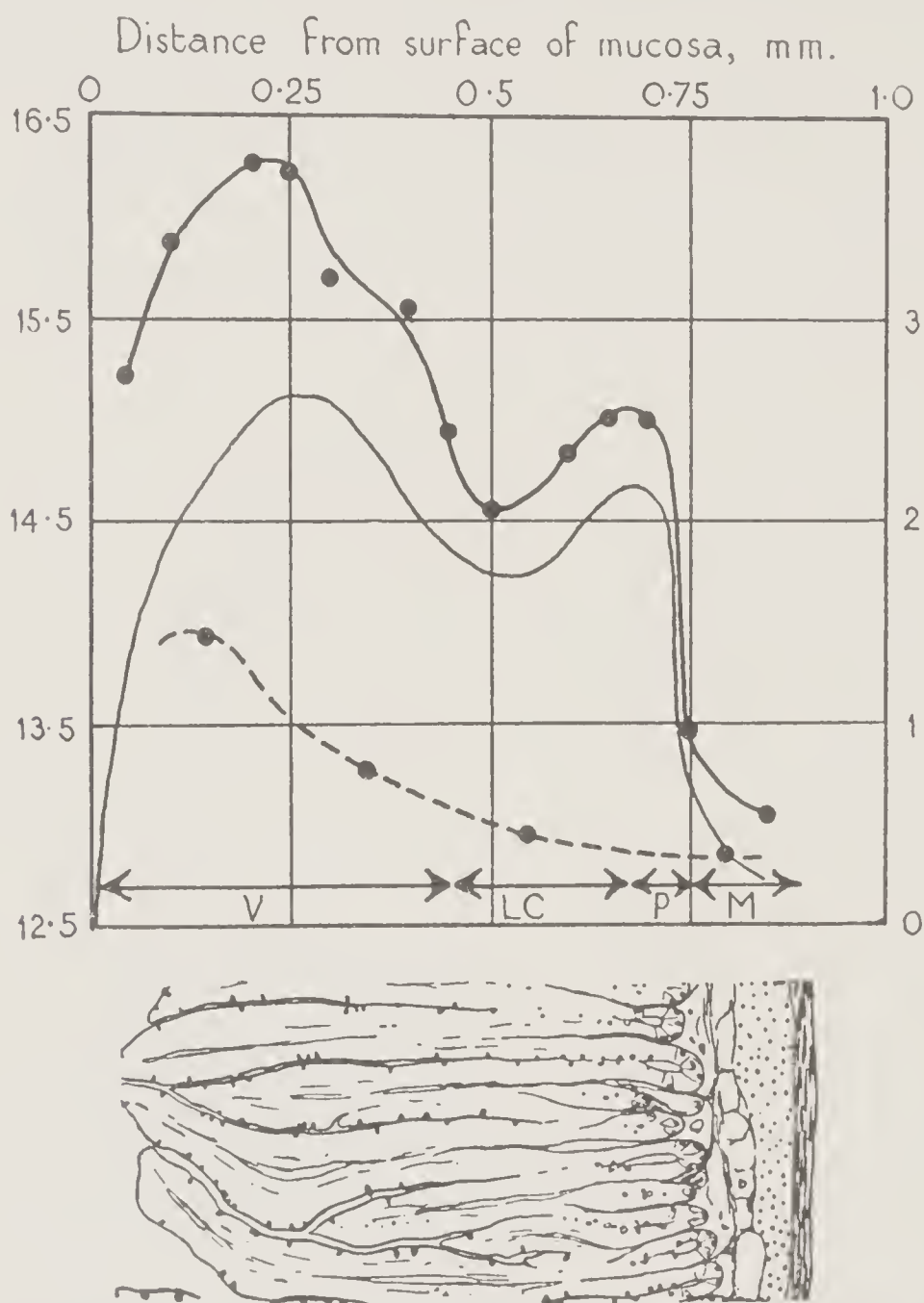
enzyme has also been studied in the ileum of the rat¹⁹⁷. Here the crypts are longer and allow a more detailed analysis, while Brunner's glands are absent. The distribution again shows two maxima, one being found in the villi, the other in the deepest part of the crypts, where Paneth



TEXT-FIG. 7. Glycyl-glycine dipeptidase activity in the upper duodenum of the rat. Values after 3 hours at 37° C. Curves: *a*, thick line: total acid used in c.mm. N/5 HCl (left ordinate scale); *b*, dotted line: blanks (left ordinate scale); *c*, thin line: difference between *a* and *b* in c.mm. N/5 HCl (right ordinate scale). The thin curve expresses the enzyme activity. The diagram shows a longitudinal section through the upper duodenum. V, Villi; LC, Crypts; B, Brunner's glands; M, Muscularis. (Van Genderen and Engel.⁷⁵)

cells occur (Text-fig. 8, and Plate II, Fig. 1). The interpretation of the facts given is that the production of the enzyme takes place in the Paneth cells, which extrude their granules into the lumen of the crypts, where they dissolve rapidly. The surface of the villi merely adsorbs the enzyme. In the light of these conclusions it would be

interesting to analyse the small intestine of dogs and cats, where Paneth cells do not occur. The granules of the Paneth cells are regenerated *c.* 7 hours after an injection of pilocarpine, but an analysis of the parallelism between the number of granules in Paneth cells and their

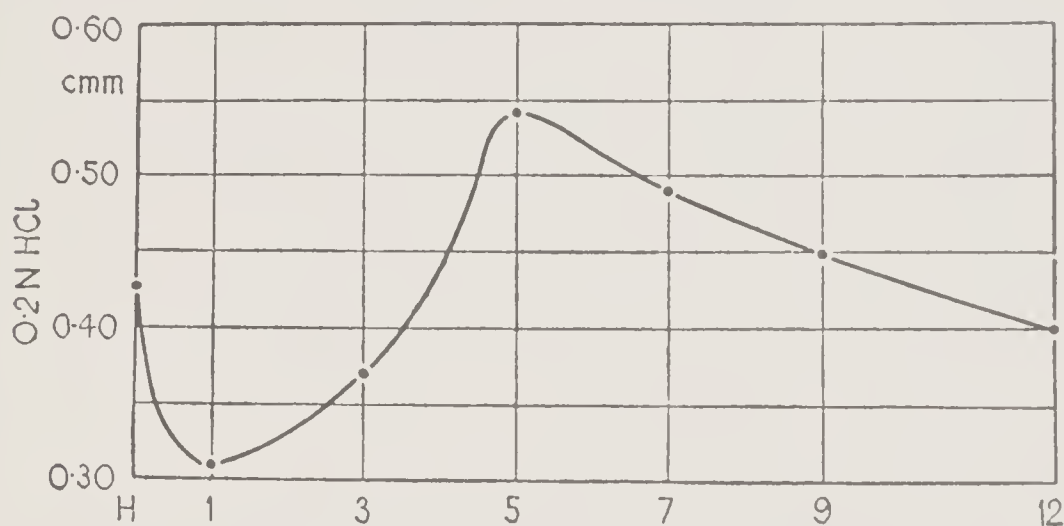


TEXT-FIG. 8. Glycyl-glycine dipeptidase activity in the ileum of the rat. Abscissae and ordinates as in Text-fig. 7. P = level of Paneth cells, other abbreviations as in Text-fig. 7. After van Genderen and Engel⁷⁵.

enzyme content does not appear to have been made; the granules themselves give a positive ninhydrin reaction⁸⁷. Even if all prismatic cells were engaged in the production of the enzyme the dipeptidase content of a single cell would be the highest so far recorded, *i.e.* *c.* 0.8 units.

(iii) *Pancreas*. Two types of peptidase, *i.e.* glycyl-glycine dipeptidase and carboxypolypeptidase, have been shown to occur in the excretory cells of the pancreas^{197,199}. The pancreatic secretion, however, contains

no dipeptidase under physiological conditions. Only if very strong stimuli are applied, e.g. intravenous injection of pilocarpine, do traces of dipeptidase appear in the secretion. This enzyme, however, can always be found in the cells. The amount of enzyme in a 25μ section (diameter 2.6 mm.) of the pancreas of a fasting rat is able to split peptide linkages corresponding to 0.4 c.mm. N/5 HCl during 2 hours

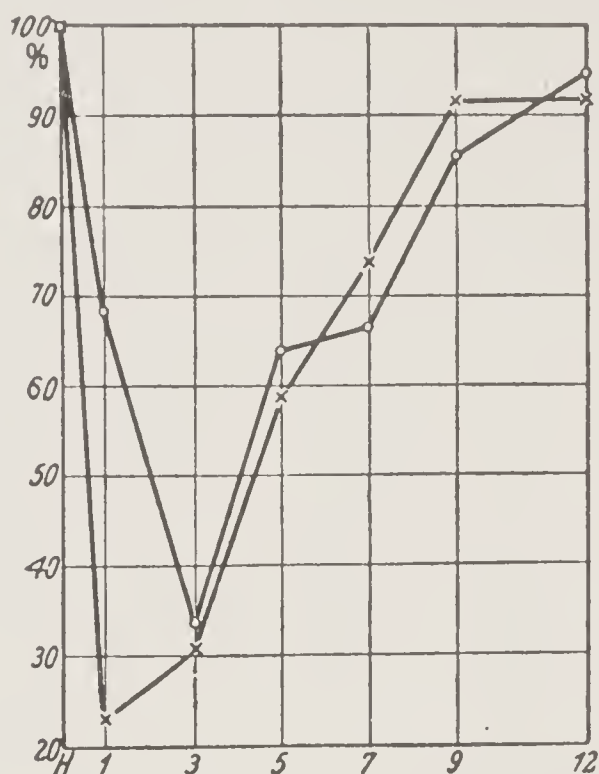


TEXT-FIG. 9. Glycyl-glycine dipeptidase activity in the pancreas of the mouse. Ordinates: c.mm. N/5 HCl due to enzyme activity. Abscissae: hours after injection of pilocarpine. H = value for the fasting animal. (Van Weel¹⁹⁷.)

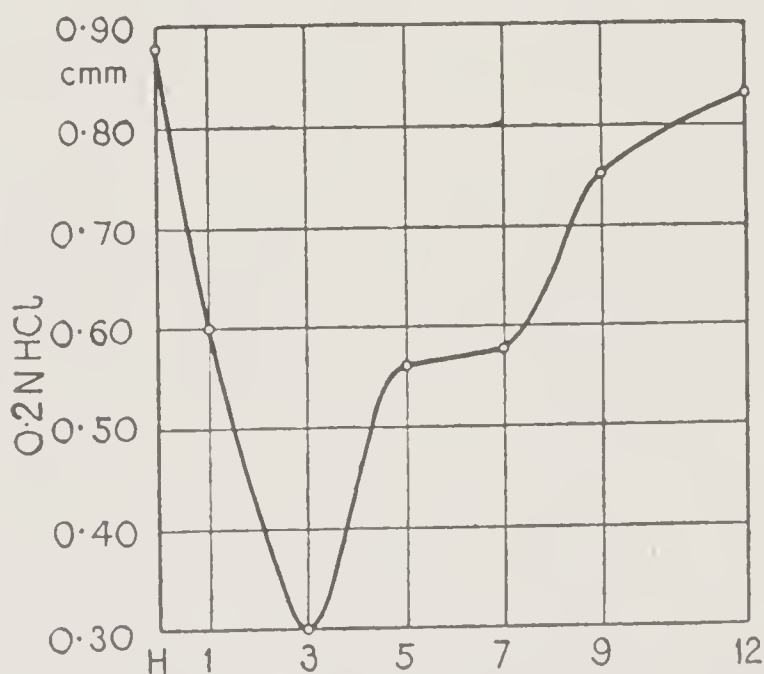
at 37° C. One hour after the injection of pilocarpine this value has dropped to 0.3 c.mm., and 5 hours after the injection it reaches a maximum of 0.55 c.mm. (Text-fig. 9). Later the activity drops gradually until it reaches the resting value 9–12 hours after the injection¹⁹⁷.

The interesting point about the regeneration of the dipeptidase is the following: the granules of the pancreatic cells are extruded into the lumen of the acini after an injection of pilocarpine; they reach a minimum in the cells about an hour later and are gradually re-formed until, after about 12 hours, the original amounts are present (curve x—x, Text-fig. 10). Dipeptidase is not present in the granules which are excreted; the presence of very small amounts of dipeptidase in the pancreatic juice after an unusually strong stimulus is due to the very rapid extrusion of the cell granules, which under these conditions carry with them small accretions of cytoplasm. This has been confirmed by cytological observation. It has been suggested¹⁹⁷ that the dipeptidase plays a part in the formation of another enzyme—the carboxypolypeptidase which is normally secreted by the pancreatic cells, and which has been prepared in a crystalline state by Anson⁶. Van Weel and Engel¹⁹⁹ were able to show that there is a definite correlation between the amount of granules in the pancreatic cells and the carboxypolypeptidase content determined in tissue sections. The highest content of this enzyme is found in a fasting animal. One hour after an injection of pilocarpine

a sharp drop occurs to two-thirds of the initial activity, and to one-third after 3 hours (Text-fig. 11). Later the enzyme content gradually



TEXT-FIG. 10. Granule content (x—x) of pancreatic cells of the mouse and carboxypolypeptidase content (o—o) of the pancreas, expressed in percentages of the values for the fasting animal (H = 100 per cent.). Abscissae: hours after injection of pilocarpine. (Van Weel and Engel¹⁹⁹.)



TEXT-FIG. 11. Carboxypolypeptidase activity in the pancreas of the mouse. Ordinates and abscissae as in Text-fig. 9. (Van Weel and Engel¹⁹⁹.)

risks again to reach its initial value after c. 12 hours. The number of granules shows a similar change. In the curves of Text-fig. 10 the number of granules and the enzyme activity of the fasting pancreas are taken as 100 per cent.; it can be seen that a striking parallelism

exists between the two curves. The time-lag in the fall of the enzyme content during the 1st to 3rd hours is due to the accumulation of secreted enzyme in the ducts of the gland, which have practically no muscle in their walls and empty their contents only slowly.

Although practically nothing is known of the synthesis of enzymes in cells, the following two points may be of interest:

- (1) By comparing Text-figs. 9 and 11 it will be seen that the rise of the dipeptidase curve, which reaches its maximum during the fifth hour, always precedes that of the carboxypeptidase curve. It is mainly on these grounds that the possible role of dipeptidase in the synthesis of carboxypolypeptidase has been suggested.
- (2) During the regeneration of the cell granules 1–2 hours after feeding, small granules are found in the cytoplasm which give a positive Feulgen reaction¹⁹⁶. Desoxypentose nucleic acid gives the same test, and possibly nucleic acids or their derivatives play a part in these processes.

d. Urease

In the mucosa of the dog's stomach this enzyme was found to be present in the prismatic cells lining the surface, in the gastric pits in fundus and pylorus, and also in the cells forming the necks of the glands in both parts of the stomach¹³⁵. The absolute values of urease activity showed considerable variation. The maxima were found in the fundus at a depth of 0.2 to 0.4 mm., corresponding to the gastric pits or the neck of the glands. The maxima in the pyloric region were at 0.4 mm. depth, corresponding to the gastric pits and the level of the necks of the pyloric glands. No urease was found in the pig's stomach¹³⁴.

e. Arginase

The distribution of arginase between the cytoplasm and the nucleus of liver cells has been studied by a number of workers^{15,185}. The method used in all cases was the fractionation of the cells by fragmentation of fresh or frozen-dried tissue followed by the separation of fractions of different density by centrifugal or flotation methods. A fraction consisting almost entirely of nuclei could be obtained in this way. The nuclear fraction had a similar arginase content to that of the other fractions. This result does not indicate that arginase is distributed evenly throughout the cell. As Danielli has pointed out, the nuclear fraction could well be contaminated by material adsorbed from the cytoplasm during the process of preparation, and vice versa.

The intracellular topography of a number of enzymes has been studied by similar methods. Only one of them (alkaline phosphatase, see below) has been shown to occur in higher concentration in the nuclei of cells. The use of such methods for determining the enzyme content of parts of cells has been discussed in an earlier chapter.

f. Phosphatases

Little is actually known about the part played by these widely distributed enzymes in cell processes, although they have been extensively investigated from a histochemical point of view. It is known, however, that they play a part in the process of ossification, and also in the synthesis of fats in the intestinal mucosa. They may play a general part in phosphorylation processes. A classification of phosphatases is given by Folley and Kay⁷¹.

1. *Intestinal phosphatase*. Jeker¹⁰¹ found that in rats poisoned with monoiodoacetic acid—a specific poison for phosphorylation processes—neutral fats fail to appear within the epithelial cells covering the villi even 6 hours after feeding with 3 c.c. olive oil, although what are probably fatty acids can be shown to be present at these sites; this suggests that a phosphatase is involved in the process.

Phosphatases capable of hydrolysing a variety of phosphorylated compounds and acting in both the alkaline and acid pH ranges have been shown in the intestinal mucosa by histochemical procedures^{27,46,47,49,57,79,103}. The methods used have been based on those first described by Gomori⁷⁸ and Takamatsu¹⁹⁰. The histochemical reaction is observed to occur in two main sites: (a) at the brush border of the epithelial cells, and within the cytoplasm of the cells, particularly in the region occupied by the Golgi element. It has been claimed that the enzymes in these two sites are differentially susceptible to KCN and to the action of different substances used as cytological fixatives. Attempts have also been made to distinguish the presence of different enzymes at these sites on a basis of substrate specificity and pH range. It is questionable, however, to what extent these enzymes are substrate-specific; they are probably more bond-specific, a single enzyme being capable of hydrolysing a large variety of monoesters of phosphoric acid¹³⁸. There is evidence, however, that the enzymes with optima in the acid and alkaline ranges represent different entities.

2. *Bone phosphatase*. Robison^{178,179} was the first to show the presence of phosphatase in bones by placing portions of bones of young rats in solutions of barium hexose monophosphate; these showed a precipitate of barium phosphate after a few hours. Later he devised a method for demonstrating the presence of this enzyme in which sections of tissue are placed in a solution containing phosphoric esters. By the action of the enzyme phosphate is liberated, to be precipitated as the calcium salt in the tissue and stained with AgNO₃ (Kossa's method) or with purpurin or other stains. This allowed a fairly accurate localization of the enzyme. This technique has since been superseded by Gomori and Takamatsu's more elegant technique, which can also be used to show bone phosphatase activity. For quantitative investigations, however, tissue extracts are used.

(a) *Amount and distribution of enzyme.* In bone, the enzyme is found wherever ossification is taking place or is going to take place. Thus the epiphysial-diaphysial junction of the growing bone has a very high content, as has also the ossifying epiphysis. Slightly lower concentrations were found in the ossifying diaphysis. Hypertrophic cartilage has also a high content of phosphatase, and it has been shown that the hypertrophy of the cartilage cell invariably precedes the ossification^{62,64}; e.g. the cells of the cartilaginous embryonic skeleton of the mandible (Meckel's cartilage) never hypertrophy, do not form perichondrial bone, and contain no phosphatase^{64,66}. The same applies to costal cartilage. By contrast, in the case of the mandibular bones, which originate as 'membrane bones', i.e. from ossification centres developing in the connective tissue independently of the cartilage, phosphatase is found only in these ossification centres. Phosphatase is also found in the ossification centre of the parietal bone, another membrane bone, and in the developing tooth.

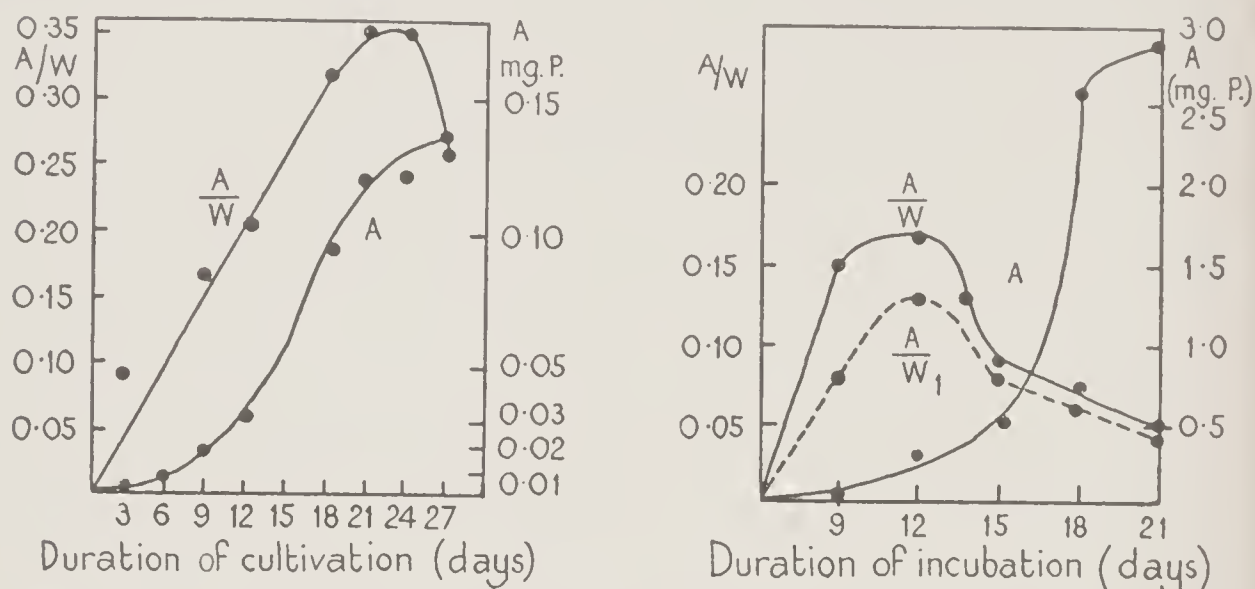
The time when the phosphatase can first be detected in the cartilage coincides exactly with the onset of hypertrophy of the cartilage cells^{64,65}. If the cartilaginous femur rudiment of the fowl embryo was explanted at an early stage (6 days) it contained no hypertrophic cartilage cells and no phosphatase. After 3 days cultivation *in vitro* the first hypertrophic cartilage cells appeared in the middle of the shaft and the first traces of enzyme could be detected (Text-fig. 12). The bone grown *in vitro* contains more phosphatase per unit weight than the bone *in vivo*. It is interesting that the enzyme is synthesized *in vitro*, independently of the blood circulation, as an intrinsic activity of the cells. Compared with other tissues the following relative phosphatase activities were found in 27-day-old rats (substrate, hexose monophosphate; pH 9)¹⁸¹:

Ossifying epiphysial cartilage of long bones	100
Costal cartilage (not ossifying)	1
Kidney	47
Liver	5
Muscle	5

The phosphatase activity of human cartilages and bones measured on 1 c.c. of 5 per cent. tissue extract for 18 hours at 30° C. at pH 8.4 (substrate, glycerophosphate) was found to be as follows:

	<i>Ester hydrolysed</i>
5½-month-old embryo: Femur shaft	60 per cent.
Epiphysial junction	71 „
Epiphysis (not yet ossified)	0.3 „
Parietal (membrane bone)	42 „
Infant full term: Costal cartilage	0 „
Costochondral junctions ossifying	59 „
Infant 7 months old: Patella: not yet ossified	0 „
Child 6 years old: Patella: half containing ossification centre	70 „
half without ossification centre	0 „

At the temperature and pH of blood the phosphatase present in a growing bone of a rabbit splits enough phosphoric esters in 1 hour to precipitate calcium phosphate equal to half the total weight of the bone.



TEXT-FIG. 12. Phosphatase content of femora of 6-day fowl embryos grown *in vitro* and during normal development (Fell and Robison⁶⁴).

Left. Production of phosphatase during the development *in vitro* of femora from 6-day fowl embryos. A = Phosphatase per femur; given as the amount of hydrolysis (mg. P) of sodium glycerophosphate in 24 hours at 37°C. and pH 8.5. A/W = Phosphatase per mg. dry weight of femur (weighed after extraction).

Right. Production of phosphatase in the femur of the embryonic fowl during normal development *in vivo*. A = Phosphatase per femur. A/W = Phosphatase per mg. dry weight of femur (weighed after extraction). A/W₁ = Phosphatase per mg. dry weight of femur (calculated on dry weight of corresponding unextracted femur).

(b) *The action of the enzyme.* It is probable that bone phosphatase is one of a group of enzymes splitting monoesters of phosphoric acid, which also includes the alkaline phosphatase demonstrated by Gomori's technique. This type of phosphatase is found in the intestinal mucosa, leucocytes, blood plasma, kidney, mammary gland, thyroid (only when inactive), and in blood vessels in the lung and central nervous system, and in capillary vessels in many parts of the body. Its activity maximum lies at pH 9.4. It is activated by Mg ions, and inhibited by M/100 KCN.

In general the enzyme is found in two main sites in the cells in which it occurs: in the cytoplasm, where it may be associated with macromolecular material¹⁵⁴, and in nuclei. A careful study of the technique by Danielli³⁹ has shown that the locations revealed histochemically probably represent the true site of the enzyme. In nuclei, the enzyme has been shown histochemically to be associated with the Feulgen-positive bands of *Drosophila* salivary gland chromosomes⁴¹. Enzyme studies on isolated nuclei have shown that alkaline phosphatase is the only enzyme which can be shown by these methods to be present in liver cell nuclei in higher concentration than in the cytoplasm⁵⁰.

Extracellularly, the enzyme is found in or on the brush borders of the cells of the epithelia of kidney and intestine, where it is presumably adsorbed⁴⁰. In hypertrophic cartilage cells the enzyme diffuses from

the cytoplasm into the surrounding intercellular spaces, where, by its action, phosphoric esters are split to bring about an increase in PO_4 ions¹⁶⁷. Two processes thus seem to be involved in the process of calcification: (1) the action of the phosphatase leading to a supersaturation of the intercellular matrix with bone salts, and (2) a mechanism leading to the deposition of these bone salts. Very little is known about this second mechanism, but it may be due to local pH changes or other factors, e.g. vitamin D. Treatment with fat solvents, desiccation, glucose, or very low concentrations of KCN impairs the second process, but not the first. In rickets a broad irregular band of hypertrophic

	<i>Hyaline cartilage</i>	<i>Hypertrophic cartilage</i>	<i>Ossification zone (or osteiod)</i>	<i>Bone of diaphysis</i>
Normal . . .	0	1.26	1.63	0.75
Rickety . . .	0	1.90	2.65	1.48

cartilage and osteoid tissue is found at the epiphysial junction. A higher concentration of phosphatase is present in rachitic bones, but calcium and phosphate cannot be deposited owing to a deficiency of the fat-soluble vitamin D¹⁷³. Moreover, fat solvents impair the deposition of bone salts in normal slices of bone (see above), and normal cartilage cells and osteoblasts contain small granules which stain with Sudan and reduce osmium tetroxide.

The importance of these other factors in promoting calcification *in vivo* is illustrated by Plate 2, Figs. 5 and 6. Here intense calcification could be shown to take place in rachitic bones *in vitro* as the result of the action of a phosphatase on calcium hexose monophosphate which could not take place *in vivo* owing to the absence of other factors.

Huggins⁹⁸⁻¹⁰⁰ has shown that when bladder epithelium of the dog is transplanted to the sheath of the abdominal rectus muscle and survives, after 18 days the surrounding connective tissue will form bone. The deposit of heterotopic bone goes parallel with the appearance of phosphatase. The following figures for enzyme activity were found:

<i>Controls</i>	<i>After transplantation of bladder mucosa in the rectus sheaths</i>
Rectus sheaths . . . 0	Rectus sheaths . . . 0.1
Bladder mucosa . . . 0.2	Rectus sheath with bladder epithelium and ossification . 0.20

So far only the ester-splitting activity of the bone phosphatase has been dealt with, but the deposition of calcium phosphate (probably as calcium carbonate apatite) proceeds in the growing bone side by side with resorption of the bone matrix by osteoclasts. Very little is known whether and at what stage of the resorption of bone-soluble phosphoric

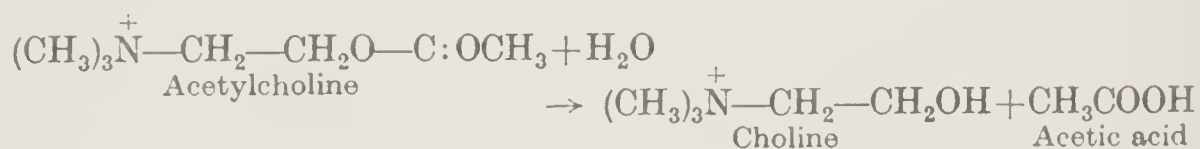
esters are resynthesized by this enzyme; but it has been shown *in vitro* that phosphatase can synthesize phosphoric esters^{145,146}. Moreover, it is known that hyperparathyroidism leads to the appearance of large numbers of osteoclasts and the removal of bone salts.

3. *Acid phosphatase*. The distribution in tissues of an intracellular phosphatase with a pH optimum of pH 4.5 and sensitivity to fluoride has been studied by a number of workers using histochemical methods⁴⁹. The sites occupied by this enzyme are, in general, different from those at which alkaline phosphatase is found. For instance, in the central nervous system concentrations of acid phosphatase are found in the cytoplasm and processes of nerve cells²³, while the latter enzyme is restricted to the glia and the vascular endothelium. However, in the hands of many workers the acid phosphatase technique has proved capricious and unreliable¹³⁸, and it has not been critically established that this technique does in fact demonstrate the true cytological localization of this enzyme (see above, Chapter I).

Moreover, the technique as at present used is at best only crudely quantitative, and does not reveal differences in phosphatase activity that can be shown by more sensitive biochemical techniques⁸⁵. Nevertheless, it has been possible to demonstrate by means of this technique that an increase in cytoplasmic phosphatase activity takes place in nerve cells during the chromatolytic cycle which follows axon section²³.

g. Cholinesterases

This group of enzymes includes several which catalyse the hydrolysis of acetylcholine:



Acetylcholine is of biological importance as a transmitter of nervous impulses at nerve endings. It was first shown by Loewi to act as the 'vagus substance' in the frog's heart, but its more generalized importance has long been recognized. The term 'cholinergic nerves' has been introduced by Dale for those nerves which act by the release of acetylcholine. In mammals, acetylcholine is believed to transmit impulses: (a) in many autonomic ganglia, (b) at the endings of many post-ganglionic autonomic fibres, chiefly in the parasympathetic system, (c) at the motor endings in skeletal muscle. Further, there are many observations—including observations on the occurrence of cholinesterase—which suggest that acetylcholine is also active in many synapses within the central nervous system. Cholinergic fibres also occur in many invertebrates: annelid worms and crustaceans have been particularly studied^{9,151,174,201}.

In 1940, Alles and Hawes described differences between the cholinesterases of the human serum and red cells⁶; the study of these differences has led to a classification of these enzymes which is not yet complete. Mendel and his colleagues¹⁴⁷ introduced the term 'pseudocholinesterase' for the enzyme, of which human and horse serum are prototypes; they have made it likely by the use of specific inhibitors that this enzyme is not concerned with destruction of transmitter in the living animal. Mendel *et al.* called the enzyme found in nervous tissue and in red cells 'true' cholinesterase, and it is this enzyme which is held responsible for the inactivation of transmitter substance *in vivo*.

Originally, 'true' cholinesterase was defined as an enzyme specific for choline esters. It has, however, been found that preparations of human red cells and of caudate nucleus are able to hydrolyse triacetin (glyceryl triacetate)²¹. Further analysis has made it likely that the hydrolysis of triacetin is in fact catalysed by 'true' cholinesterase^{3,18,97}. It is of interest that both acetylcholine and triacetin are also hydrolysed by cobra venom²⁸; here again it appears that one and the same enzyme is responsible for the hydrolysis of both acetylcholine and triacetin¹⁹; this suggests that a preference for acetates seems a general property of this type of enzyme.

The 'pseudocholinesterase' type of enzyme is also widely distributed. Purified preparations of the enzyme have been obtained from horse serum and dog pancreas. High cholinesterase activity in the mucous membrane of the stomach was described before the separate entity of the different cholinesterases was recognized; using the methods developed by Linderström-Lang, Glick⁷⁷ has shown that the activity is particularly concentrated in the region of the gastric surface cells. Recent observations show that this enzyme is to be classified as a 'pseudocholinesterase' (Blaschko and Hawkins unpublished work).

It must not be forgotten that substrate specificity is determined by the character of the enzyme protein, and that it is therefore probable that the cholinesterases of different species and from different organs will be different in some respects. This is true for some of the cholinesterases from invertebrates, some of which display interesting differences in the pattern of substrate specificities⁷. In addition to the enzymes mentioned there exist cholinesterases which are inactive towards acetylcholine, but are able to hydrolyse other cholinesters, e.g. benzoylecholine. An enzyme of this type is found in ruminants, e.g. in the kidney of the ox; another occurs in the guinea-pig's liver.

The distribution of cholinesterase in the nervous system has been well studied¹⁶³. The distribution of the enzyme is compatible with the view that it is responsible for the inactivation of the transmitter after it has done its work. It is obvious that a substance which has such powerful effects must be prevented from spreading and stimulating

distant excitable structures. In addition, the transmitter must be prevented from stimulating for too long a period.

The distribution of the enzyme, as studied in the mammal, corresponds to these functions: concentrated wherever cholinergic fibres innervate an excitable structure with a short refractory period, e.g. in striated muscle or in the sympathetic ganglion. In the case of striated muscle the distribution of cholinesterase in the sartorius of the frog (*Rana esculenta*) has been studied. This muscle was chosen because the distribution of nerve endings in the muscle has been well studied. The muscle is known to be free of nerve endings in its pelvic end. A careful study of the distribution of nerve endings was recently made by Pézard and May¹⁷¹. They applied a modification of Ramon y Cajal's silver method and made serial sections of the muscle and counted the nerve endings. They found that the pelvic end—about one-eighth of the length of the muscle—was completely free of nerve endings, the pelvic fifth contained only very few endings, but the adjoining fifth of the muscle was extremely rich in endings. The distribution of cholinesterase was determined as follows (Marnay and Nachmansohn¹⁴⁴): the muscle was divided into five parts, the tissues were finely ground, and the esterase activities of the different portions determined. The extracts were suspended in a bicarbonate solution and acetylcholine was added. The acetic acid formed in the hydrolysis of acetylcholine liberated CO₂ from the solution; the rate of CO₂ formation was determined manometrically. The activities are expressed as QChE, i.e. as mg. acetylcholine chloride hydrolysed by 100 mg. of tissue in 60 minutes. For the two adjoining portions the following QChE values were found:

Pelvic end: 0.135. Second fifth: 0.795.

In order to know the relative concentration of the enzyme in or around the nerve endings, it would be necessary to have data about the volume of the motor endings, but it is obvious that the concentration of the enzyme at or around the endings must be considerable. (The authors give arguments which show that the increased activity in the portions containing the endings cannot be due to the presence of the nerve fibres.) The localization of cholinesterase in mammalian muscle has been studied by Couteaux and Nachmansohn^{38,39}). The guinea-pig's gastrocnemius was frozen and sections were used for histological examination and determination of esterase activity. The distribution of the terminal branches of the nerve in the muscle makes it possible to obtain end-plate-free sections and sections rich in end-plates. The latter sections have the higher esterase activity.

In the sympathetic chain of the cat, the QChE of the superior cervical ganglion was 45 to 60, that of the afferent fibres in the sympathetic nerve about 5. Similar differences occur in the abdominal chain of the

lobster, where the ganglia contain more than twice the amount of activity of the fibres¹⁵⁹⁻¹⁶¹.

The electric organ of the ray, *Torpedo marmorata*, is generally considered as a collection of modified motor end-plates. Determination of the cholinesterase activity of the organ showed that the organ has a very high cholinesterase activity. It was this finding which led to an analysis of the nerve supply to the electric organ by Feldberg, Fessard, and Nachmansohn⁵⁹, in which the cholinergic nature of the innervation was established: if the organ is made to discharge by electrical stimulation of its nerve, acetylcholine can be discovered in the perfusion fluid, and close-range arterial injection of acetylcholine causes a discharge of the organ. The esterase activity in the electric organs of different species varies. This is shown in the following table¹⁶⁰.

Species	QChE
<i>Raja</i> . . .	3-10
<i>Gymnotus</i> . . .	90-150
<i>Torpedo</i> . . .	150-300

The electric organ which produces the highest voltages is that of the electric eel, *Electrophorus electricus*, a South American species; values of QChE up to 500 have been measured in the electric eel. The correlation between voltage and enzymic activity has also been studied; a parallelism between the voltage developed and the enzymic activity has been established¹⁶³.

The esterase content of the mammalian brain has also been studied. It was found that the esterase is very unevenly distributed. The white matter is poor in esterase activity; more of the enzyme is found in the grey matter, but the activities differ greatly in different parts. The following table gives data for cholinesterase activities as found in the ox brain.

	QChE
White matter	0.2-0.3
Cortex	2-3
Caudate nucleus	40-43
Lenticular nucleus (putamen)	68-70
Cerebellum	2.5-4
Thalamus	2.5-5
Corp. quadrigem. ant.	9.5-13
.. .. post.	4

The reason for the uneven distribution of the enzyme in the grey matter is unknown. That the enzyme is connected with the function

of the central nervous system is made likely by the study of enzymic activity during development. The esterase activity of the chick's brain is already high at the time of hatching. At that time most of the synapses in the brain must already be functioning. In mammals characteristic differences are found in different species: in the guinea-pig, an animal which is already well developed at the time of birth, the rise in esterase activity during development occurs chiefly before birth; in animals like the rat, the cat, and the rabbit, which are born in a much less mature stage, the final figures for esterase activity in the brain are only attained some time after birth. In sheep embryos the esterase activity in the spinal cord is high at a time when reflex muscular movements occur (60 to 80 days)¹⁶¹.

The high concentration of cholinesterase in the neighbourhood of synapses raises the question as to the localization of the enzyme in the nerve. This cannot be studied in the nerve fibres of vertebrates which are too small to be subdivided, but in the giant axon of the squid it has been possible to squeeze out the axoplasm and to study the enzymic activity of the extruded axoplasm and of the remaining sheath. It was found that most of the activity was concentrated in the sheath²⁴. The high cholinesterase activity of the synapses was therefore interpreted as due to increase of surface in the region of nerve endings.

These last observations have led Nachmansohn to extend the study of the function of cholinesterase to the conduction of the nervous impulse along the axon. It was found that inhibitors of cholinesterase (e.g. eserine and diisopropylfluoronophosphonate) abolished the axon potential of the giant axon when the inhibitor concentration in the fibre was sufficient to abolish cholinesterase activity almost completely. On the other hand, anticholinesterases, like prostigmine, with a poor solubility in fats, did not penetrate the nerve membrane: they did not reach a concentration in the fibre sufficient to inhibit cholinesterase and they did not abolish the action potential. These observations have led to the suggestion that, as in transmission, cholinesterase activity is also essential in the nerve fibre for the conduction of the impulse (Nachmansohn¹⁶³).

The cholinesterase content is also diminished after degeneration. This was first studied by von Brücke³² for the superior cervical sympathetic ganglion of the cat. In quantitative studies, Couteaux and Nachmansohn^{38,39} found the final value in the ganglion after degeneration still 4 to 5 times that of normal fibres. Recent observations by Sawyer and Hollinshead¹⁸⁴ suggest that this residual enzymic activity in the ganglion is due to the presence of 'pseudocholinesterase'; this activity is lost more slowly on degeneration than the 'true cholinesterase' activity.

In skeletal muscle there occurs an apparent increase of cholinesterase on section of the motor nerves, but this is due to the decrease in the

total volume of the muscle during degeneration. Using frozen sections, as described above, it has been shown that the nerve endings lose some of their activity, but that the activity remains relatively high.

A histochemical method for the detection of cholinesterases in frozen sections has been described by Koelle^{113a}. This is based on the ability of the enzyme to act upon esters of thiocholine (e.g. acetylthiocholine or butyrylthiocholine). The thiocholine set free in the enzymic reaction is precipitated by cupric ions and can thus be made visible. The specific ('true') and non-specific ('pseudo') cholinesterases can be distinguished: specific cholinesterase is shown up by using acetylthiocholine as substrate in the presence of 10^{-6} M diisopropylfluorophosphonate (DFP), which selectively knocks out the non-specific cholinesterase at this concentration; butyrylthiocholine is used as a substrate of the non-specific cholinesterase, and it serves to demonstrate the presence of this enzyme.

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DESCRIPTION OF PLATES

PLATE 1

FIG. 1. Microphotograph ($\times 800$) of eosinophil secretory cells from the gill leaflets of the eel. (After Keys and Willmer¹¹³.)

FIG. 2. Villus of small intestine of rat, 30 minutes after feeding 3 c.c. olive oil, showing fatty acid in the epithelial cells as black droplets (Fischler's method). (Jeker¹⁰¹.)

FIG. 3. Villus of small intestine of rat 6 hours after feeding 3 c.c. olive oil, stained as in Fig. 2. The epithelial cells are filled with droplets which remain unstained but are Sudan positive. The lipase has resynthesized neutral fats from fatty acids. Only a few fatty-acid droplets (black) remain in the cells. (Jeker¹⁰¹.)

FIG. 4. High-power drawing of an epithelial cell at the stage of Fig. 2.

FIG. 5. High-power drawing of an epithelial cell at the stage of Fig. 3.

PLATE 2

FIG. 1. Crypt of Lieberkühn ($\times 900$) showing three Paneth cells with their specific granules at the bottom of the crypt, also two goblet cells (S). Mitochondria (Mit.) and Golgi apparatus (black threads) in all cells. (Van Weel¹⁹⁷.)

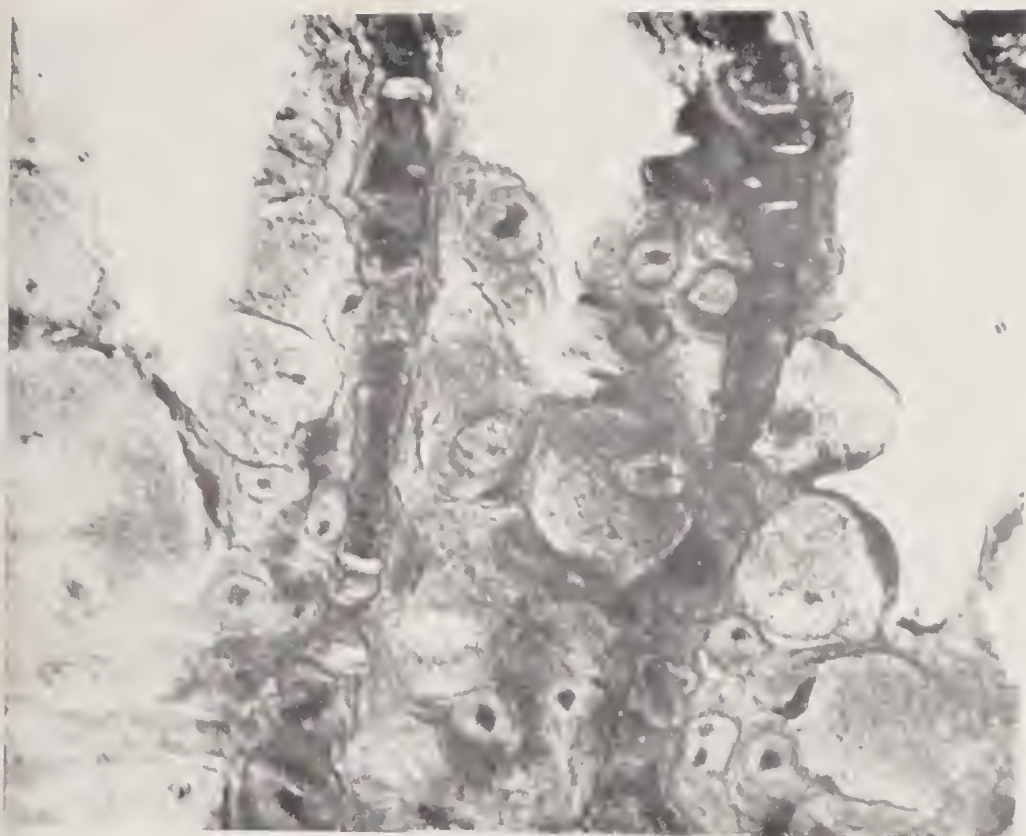
FIG. 2. Villus of small intestine of rat, 7 hours after a dose of monoiodoacetic acid which was followed 1 hour later by 3 c.c. olive oil, stained by Fischler's method. The black droplets of fatty acid are still found in the epithelium; some have diffused into the stroma. Synthesis of neutral fats has been blocked. Compare with Plate 1, Fig. 3. (Jeker¹⁰¹.)

FIG. 3. Longitudinal section of a tibia of an 18-day rabbit embryo. Calcification black by treatment with AgNO_3 , nuclei counterstained with Carmalum. $\times 55$. The diaphysis is composed of hypertrophic cartilage, partly calcified (C.H.C.), and a small layer of periosteal bone (C.O.) on one side. At the other side the periosteum has not yet formed bone (U.O.). Z.F.C. Zone of flattened cartilage cells. $\times 55$. (Niven and Robison¹⁶⁷.)

FIG. 4. The other tibia of the same embryo, immersed for 22 hours at 37°C . (pH 7.4) in a solution containing 8 mg. Ca, 4 mg. inorganic P, and 3 mg. organic P (as glycerophosphoric ester) in 100 c.c. Note heavy disposition of calcium salt in the matrix of the hypertrophic cartilage and in the periosteal layer by the action of the phosphatase. Stained as in Fig. 3. $\times 55$. (Niven and Robison¹⁶⁷.)

FIG. 5. Epiphysial-diaphysial junction of the radius of a ricketic rat, after 16 hours' immersion in 0.1 M sodium phosphate at 37°C . Note the broad band of hypertrophic cartilage (h.c.) that has remained uncalcified, and also the incomplete ossification of the osteoid trabeculae; e.c., small cell cartilage. (Robison¹⁸⁰.)

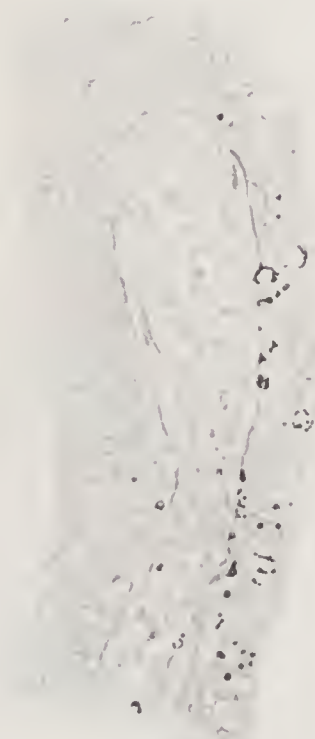
FIG. 6. The same region of the other radius of the same rat, after 16 hours' immersion in 0.1 M. calcium hexose-monophosphate, as the only source of phosphate. By the action of the phosphatase the hypertrophic cartilage and the osteoid trabeculae are heavily calcified. The small cell cartilage (e.c.) remains uncalcified. (c.h.c. calcified hypertrophic cartilage.) (Robison¹⁸⁰.)



1



2



3



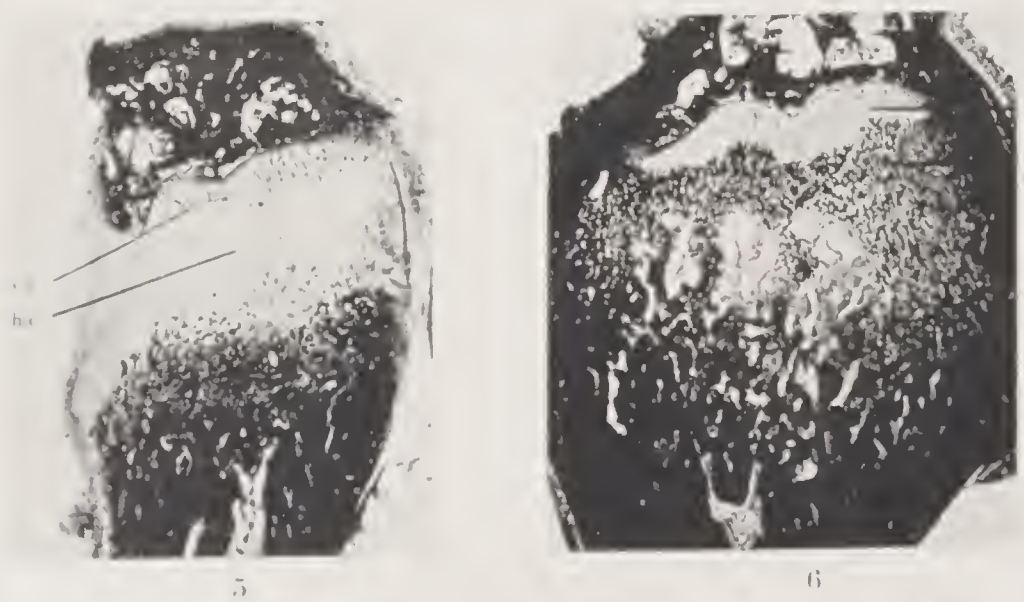
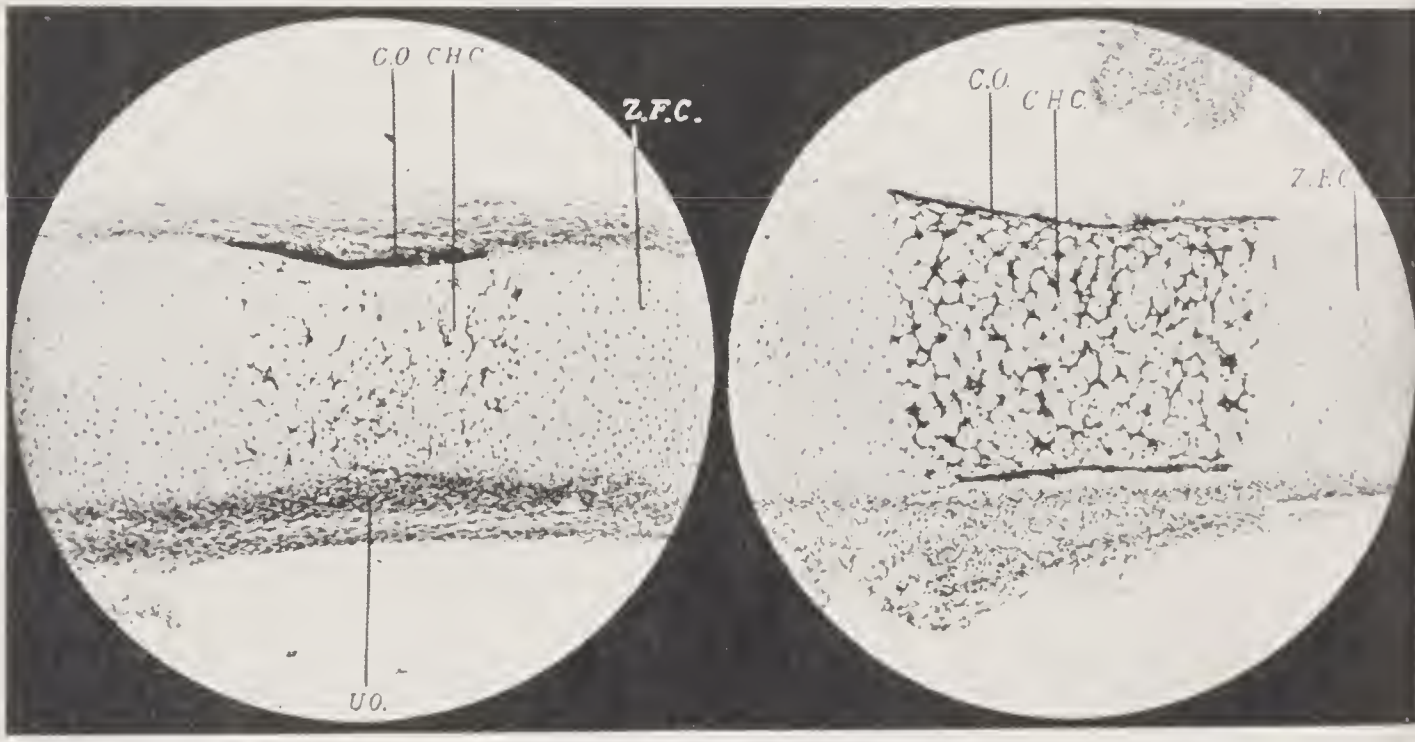
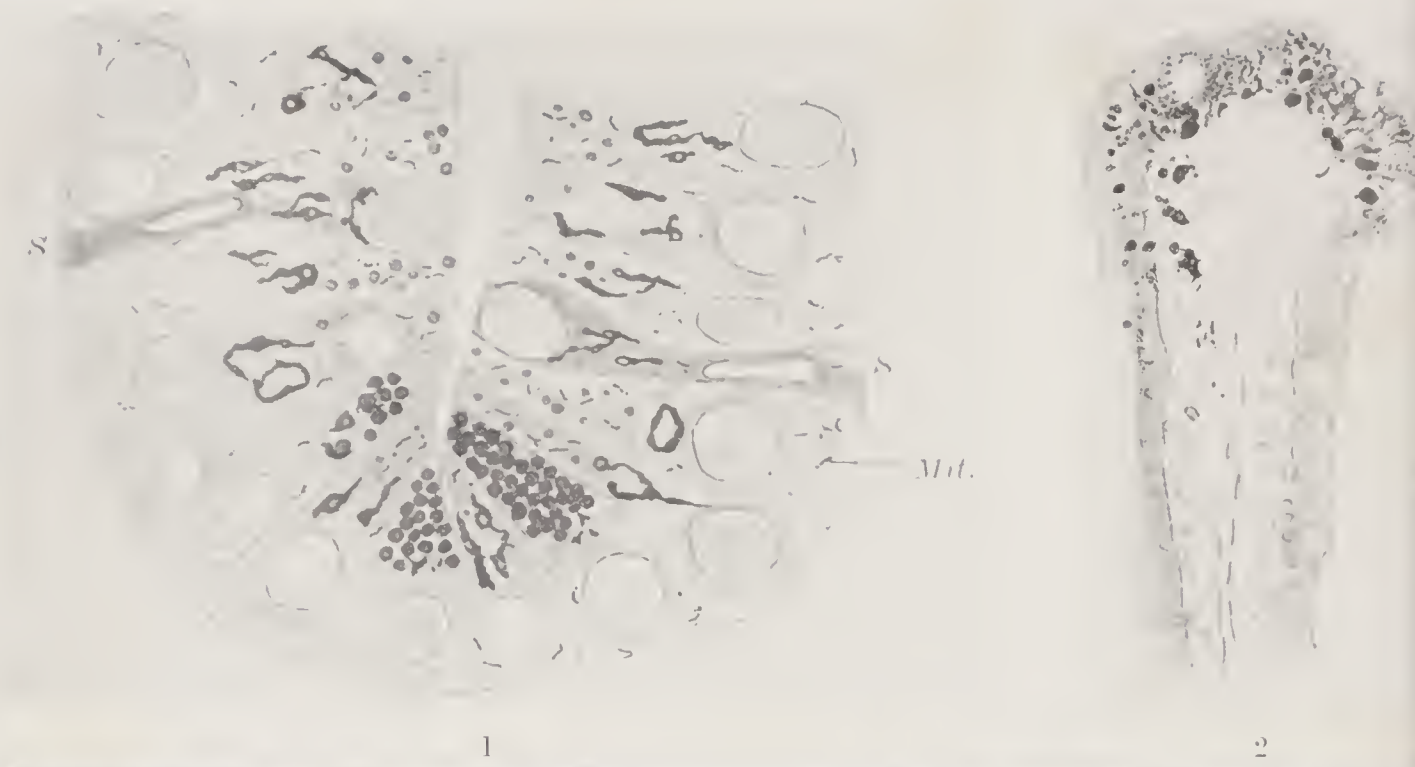
4



5

FIG. 1. Eosinophil cells from the gill leaflets of the eel. FIGS. 2-5. Action of lipase in intestinal cells. *For description see p. 371*

H. Blaschko and W. Jacobson



Enzymes of intestine and bone. For description see p. 371

CHAPTER IX

PATHOLOGICAL ASPECTS OF CYTOLOGY

By R. J. LUDFORD

i. INTRODUCTION

THE structure of cells varies with their state of functional activity. The range of variation in cellular structure is well illustrated by gland cells in various phases of activity. Precise morphological differences characterize 'resting' and dividing gland cells, and those engaged in the elaboration of their specific secretions. Such changes are the expressions of the normal range of physiological functioning. Other modifications of cell structure accompany aberrations of functioning, are the result of alterations in the humoral environment, or are consequent upon infections. These latter constitute the pathological modifications of cellular structure.

ii. REGRESSIVE CHANGES

Cellular degeneration is the most frequent result of pathological conditions. The morphological changes which occur in degenerating cells are most conveniently studied in living tissue cultures, especially in those prepared with fluid culture media. Under such conditions well-spread cells can be obtained on the surface of cover-glasses. They are best examined by washing off the culture medium and mounting in a saline solution. Such preparations are ideal for examination by dark-ground illumination, which permits of the most detailed observation of changes in protoplasmic structure.

When a cell from an actively growing culture of mammalian fibroblasts is examined in this way (Plate 5, Fig. 1) the only nuclear structures clearly distinguishable are one or more nucleoli and the nuclear membrane; but in the cytoplasm the mitochondria are conspicuous objects. There are also usually to be seen a few minute cytoplasmic granules which stain readily with neutral red, and a few small, highly refractile fat droplets. What is generally described as the 'ground cytoplasm' is optically empty. Centrioles are not distinguishable, nor is the Golgi apparatus. This does not necessarily imply that the Golgi apparatus as demonstrated by the osmic or silver-impregnation techniques is an artefact. It is probable that its refractive index does not differ sufficiently from that of the surrounding cytoplasm to render it visible.

When cultures are prepared and simply incubated, without receiving any further treatment, conditions soon become unfavourable for growth, owing to the accumulation of toxic metabolic products and depletion

of essential nutritive requirements. There is thus initiated a progressive series of cellular changes, often continuing slowly for several weeks before terminating in the death of the cells. It will be convenient to discuss separately the changes occurring in (a) the mitochondria, (b) the ground cytoplasm, (c) the sphere, (d) the Golgi apparatus, and (e) the nucleus.

a. *The Mitochondria*

The mitochondria are particularly sensitive to pathological conditions. In actively growing cells they tend to be mostly filamentous and spread throughout the greater part of the cytoplasm. With the advent of adverse conditions they begin to collect around the nucleus, usually becoming heaped up around the central body. Filaments break up into granules, and some swell and become vesicular, as seen in Plate 1, Fig. 1. In advanced stages of degeneration the mitochondria are reduced to fine granules, scattered amongst other cytoplasmic particles, with usually a few short rod-like forms at the extreme periphery of the cell. They still retain their specificity for the vital dyestuffs, Janus green and Janus black, which renders their identification possible. Finally, they appear to disintegrate completely.

Under certain pathological conditions *in vivo* mitochondria have been reported to undergo fatty changes. In a fat-cell tumour (liposarcoma) of the guinea-pig Murray¹⁷⁷ described the mitochondria swelling up and becoming converted into fat droplets. In degenerating tumour cells the fragmented mitochondria also appear to give rise to fat droplets (Ludford¹⁴⁴). Scott²¹⁴ found that the filamentous mitochondria of acinar cells of the pancreas became granular in phosphorus poisoning. The granules fused together and gave rise to fat droplets. According to Kater¹⁰³ fat droplets arise within the mitochondria of liver cells. As such fat droplets enlarge they are surrounded by an incomplete peripheral film of mitochondrial substance. Duthie⁶², however, disagrees with this. He describes the mitochondria of degenerating liver cells as becoming enspherulated and enlarged, then vacuolated, and finally disappearing without forming fat. A similar process was described by Ostrouch¹⁸⁹ as being brought about by the action of ethyl alcohol on epithelial cells of the stomach. The mitochondria became granular, lost their polarized distribution above and below the nucleus, enlarged, and then disappeared as the cytoplasm became vacuolated. According to Grynfeltt and Lafont⁸⁷ the mitochondria of liver cells became granular with sulphonal poisoning and were displaced towards the periphery of the cells.

Although mitochondria of most cells are particularly sensitive to pathological conditions, those of nerve cells seem to be exceptional. Cowdry⁴³ cites as examples of their stability under pathological condi-

tion the retention of their normal appearance in nerve cells injured by the viruses of poliomyelitis (McCann¹⁷¹) and herpetic encephalitis (Cowdry and Nicholson⁵¹).

Phase-contrast microscopy has opened up new possibilities for the study of mitochondria in living cells. Zollinger²³⁷, who studied living cells of kidney tubules, observed mitochondria become granular and swollen. Isolated mitochondria from crushed cells in saline solutions appeared vesicular, as though a peripheral membrane had become distended by intake of fluid. He suggested that 'cloudy swelling' resulted in this way. Ludford, Smiles, and Welch¹⁶¹ found that mitochondria of malignant cells react rapidly to ultraviolet radiations of wave-lengths of 2,570 Å and 2,750 Å. They first become distorted, then disintegrate, leaving vacuoles in the cytoplasm. Zollinger pointed out that although mitochondria react readily when cells are exposed to adverse environmental conditions they are not fragile, since they persist for some time after cells have been squashed in saline solutions.

b. The Ground Cytoplasm

Although the ground cytoplasm of healthy fibroblasts growing in tissue cultures is optically structureless, it often contains a few granules which stain with neutral red and other basic dyes such as methylene blue and cresyl blue. Lewis¹¹² calls all such neutral-red-staining particles 'degeneration granules'. Carrel and Ebeling²³, however, consider a juxta-nuclear group of neutral red granules to constitute a 'segregation apparatus', which enlarges by increase in number and size of the constituent granules with increased rate of cell growth. Rumjantzew²¹² also supported this conception of a segregation apparatus. Both Lewis¹¹⁶ and Ludford¹⁵³, however, found few or no neutral-red granules in young, actively growing fibroblasts, but observed their accumulation as cultures deteriorated. They accumulate around the sphere, spreading out towards the periphery of the cell. Lewis¹¹⁶ observed the formation of vacuoles about these granules; but they may fill almost the whole of the cytoplasm without any large vacuoles being formed, as can be seen in the binucleate cell of Plate 1, Fig. 4. It is in such cells as this that one often sees a clear reticulate area of cytoplasm which bears a striking resemblance to the Golgi apparatus of fixed tissues.

In addition to the formation of degeneration granules, there may also be considerable vacuolation of the cytoplasm (Plate 1, Fig. 3) and formation of fat droplets. All three processes may occur concurrently in the same cell, but neighbouring cells in the same culture may exhibit very different degenerative changes. Lewis suggested that the degeneration granules and associated vacuoles are the accumulations of the waste products of autolysis. He found that the same sort of cytoplasmic granules and vacuoles occur when cells degenerate in the bodies of dead

animals as when they are incubated in saline *in vitro*. There is, however, considerable difference in the rate at which different cells degenerate and die. Thus, macrophages survive for about 10 days and brain cells for less than an hour (Lewis and McCoy¹²⁴). Experiments on cytoplasmic viscosity led von Möllendorff¹⁷⁴ to the conclusion that vacuole formation is a cellular defence mechanism against substances injurious to cells. Compounds such as potassium chloride and potassium acetate, which lower cytoplasmic viscosity and increase the rate of movement of cytoplasmic granules, subsequently bring about vacuole formation at the expense of the granules, thereby restoring the normal viscosity. The acid vital dyestuffs, colloidal particles, and fat are alike segregated in vacuoles and so prevented from interfering with cellular metabolism. The basic vital dyestuff neutral red, when applied to living cells, first colours the cytoplasm diffusely, but is soon flocculated on the surface of granules and vacuoles. During the phase of diffuse staining mitosis is inhibited, but can proceed after the dyestuff has been segregated, if the excess dyestuff is washed away. Mitosis can proceed quite normally in cells containing numerous fat droplets. Nagel¹⁸¹ has drawn attention to the fact that cells containing many vacuoles which stain vitally with basic dyestuffs have few fat droplets, and vice versa. Fat droplets also tend to collect around the sphere like the 'degeneration granules' and vacuoles (Plate 1, Fig. 2). The origin of the fat appearing in cells *in vitro* has been the subject of much discussion, in connexion with the problem as to the source of fat in cases of fatty degeneration of tissues. With cells growing *in vitro* the extent to which they become laden with fat droplets depends to a large extent upon the medium (Lambert¹⁰⁸). The addition of yolk to the culture medium results in the appearance of fat in the cells (Lewis¹¹¹). Rat and mouse cells develop more fat droplets in mouse serum than in the less fatty rat serum (Ludford¹⁵⁰). The extensive accumulation of fat in degenerating cells described by Horning and Richardson⁹⁷ is a characteristic feature of chick tissues grown in fowl plasma, and contrasts strikingly with the degenerative process occurring in rat cells grown in rat serum, in which there is little or no fat formation. It is clear, therefore, that fat may be of extracellular origin and be segregated in droplets in the same way as are other foreign substances. Dible and his collaborators have adduced evidence in a series of researches that the accumulation of fat droplets in cells of the liver, heart, and kidney under various pathological conditions is always associated with an increase in the fat content of these organs, and that this excess results from infiltration of the cells. Furthermore, this fatty infiltration is dependent upon adequate fat in the animal's storage deposits (see Dible and Gerrard⁵⁹; Dible and Hay⁶⁰). Other investigators have contended that fat droplets originate from the disorganization of the cytoplasmic colloids (phanerosis). There has been

much discussion as to whether proteins and lipides exist in the cytoplasm in a state of physical or chemical combination. From his critical survey of the literature Kiessel¹⁰⁵ concluded that the two types of substances form adsorption complexes rather than chemical compounds. Pollack's¹⁹⁸ experiments, in which he precipitated protein from aqueous solutions of dead amoebae with picric acid, but on injecting the same solution into the living organisms obtained no precipitate, raises doubts as to whether proteins, as such, exist in living cells. According to Nadson¹⁷⁸, Nadson and Meisl¹⁷⁹, and Nadson and Rochlin¹⁸⁰, in plant cells separation of lipides and proteins is brought about by irradiation and chloroform poisoning, and results in the formation of fat droplets, proteins being irreversibly coagulated at the same time.

Cells in tissue cultures exhibiting early degenerative changes are able to recover if they are transplanted to fresh culture media. Contact of fibroblasts with cultures of monocytes and macrophages also has a rejuvenating effect, leading to the disappearance of fat droplets and cytoplasmic granules, and the stimulation of growth (Carrel and Ebeling²¹; Ludford¹⁵⁷). The final changes of cell degeneration *in vitro* involve what remains of the ground cytoplasm, which becomes filled with very fine granules, best seen by dark-ground illumination. If the cells have been vitally stained the colour diffuses from the mitochondria and from the 'degeneration granules' and vacuoles. The nuclear membrane becomes more clearly defined, the nucleolus is coloured, and the fine granulation spreads throughout the nucleus. Any vital dye present stains the whole cell diffusely. The cell colloids are now irreversibly coagulated.

c. *The Sphere or Central Body*

In healthy, actively growing cells *in vitro* the central body is not a clearly defined structure, although the mitochondria tend to collect at one side of the nucleus, where in fixed and stained preparations the centrioles are distinguishable. Mitochondria move backwards and forwards from this juxta-nuclear area to the cell periphery. When 'degeneration granules' make their appearance they accumulate in this area. Later, in the midst of the group of granules a clear area appears, and it is in the centre of this that the centrioles are demonstrable after fixation and staining. Filamentous mitochondria usually become radially arranged around it. In some cells this central area undergoes a remarkable hypertrophy, resulting in a so-called giant centrosphere, which may be larger than the nucleus. According to Lewis¹¹³, in its complete form it consists of a centriole contained within a medullary zone which stains deeper than an external cortical zone, and from which it is separated by a thin membrane. Lewis¹¹⁷ has observed giant centrospheres only in cultures of mesenchyme, mesothelium, and endothelium.

The origin of the substance forming these large bodies remains obscure. Lewis¹¹³ suggests that the clear area around the centriole results from the accumulation of some of the cytoplasm flowing centripetally towards it. This streaming would account for the radial arrangement of the mitochondria. The material thus accumulated probably acquires a more solid consistency, thus preventing mitochondria and 'degeneration granules' from penetrating into it, and pushing them outwards as it enlarges. These giant central bodies are of interest owing to their similarity to certain inclusions that occur in some malignant cells, and which at the time of their discovery were mistaken for the causative organisms of malignant disease. Of these inclusion bodies it now seems quite clear that some were enlarged central bodies, and others products of abortive secretory activity (Ludford¹³⁴).

d. The Golgi Apparatus

Reference has already been made to the fact that a reticulate area resembling the Golgi apparatus is visible in some degenerating cells (Plate 1, Fig. 4). A similar colourless network can also be demonstrated under certain conditions in cells, the cytoplasm of which has been diffusely stained with methylene blue (Ludford¹⁵²). A reticulate structure has also been observed in some living cells when examined by the phase-contrast microscope (Ludford, Smiles, and Welch¹⁶²). In fixed tissue cultures suitably impregnated by either the osmic or silver techniques, the Golgi apparatus has been demonstrated in a variety of normal and malignant cells (Ludford¹³⁵; Zweibaum and Elkner²⁴⁰; Vázquez López²³⁰; Saguchi²¹³; Richardson²⁰³; Macdougald and Gatenby¹⁶⁶; and Macdougald^{164,165}). Apart from Zweibaum and Elkner, who accepted the Parat conception of a zone of Golgi consisting of 'chondriome actif' and 'vacuome', the other investigators are in agreement that the Golgi apparatus is a cytoplasmic structure independent of neutral-red-staining vacuoles. There is also general agreement that the Golgi apparatus breaks up into granules in degenerating cells. Ludford's observations led to the conclusion that the fragmented particles of the Golgi apparatus gave rise to fat droplets. Richardson confirmed this observation, as did also Macdougald, and Macdougald and Gatenby. In a later paper, however, Macdougald¹⁶⁵ denied that the brown droplets resulting from the fragmentation of the Golgi apparatus in osmic preparations were fat, since they did not stain with Sudan III.

Fragmentation of the Golgi apparatus has been described under a variety of pathological conditions: during autolysis of normal and malignant cells (Cajal¹⁹; Ludford¹⁴⁴); in kidney tubules in experimental nephritis (Pappenheimer¹⁹⁰); in nerve cells following section of axons (Penfield¹⁹⁵); in cells subjected to the action of poisons, and following irradiation. A considerable enlargement of the apparatus, with thicken-

ing of its strands, unaccompanied by secretory activity, occurs in acinar cells of the pancreas after repeated injections of neutral red (Ludford¹⁴³). Such cells present the appearance of having had their secretory mechanism blocked by the accumulation of the dyestuff.

The sensitivity of the Golgi apparatus to injury probably varies in different types of cells, as is the case with mitochondria. It is of interest to note that in studying the effect of phosphorus poisoning and irradiation on the spermatogenesis of *Abraxas*, Gatenby^{75,76} found that the Golgi apparatus was the most sensitive of the cell organs.

e. *The Nucleus*

The degenerative changes occurring in the nuclei of cells growing *in vitro* have not received the same attention as the cytoplasmic ones. As already described, the cytoplasm can be studied by the aid of vital staining, and finer changes in its structure revealed by dark-ground illumination. Neither of these techniques is suitable for the study of the nucleus. Vital dyes do not penetrate the nucleus until the cell has reached an advanced stage of degeneration or is subjected to special physiological conditions (Ludford¹⁴⁷), and dark-ground illumination only reveals clearly the nucleolus and nothing of the chromatin.

Nucleolar extrusions into the cytoplasm have been described in cultures of endothelium (Lewis¹¹⁵) and fibroblasts (Ludford¹³²). As the same process has been reported in many different types of cells, under varying physiological conditions *in vivo*, it is doubtful whether its occurrence in tissue cultures is indicative of pathological change. The formation of nuclear buds which become nipped off and enclosed within cytoplasmic vacuoles is an early indication of abnormality. They remain for a time in the cytoplasm, resembling somewhat the secondary nuclei of Hymenopteran oöcytes, but ultimately they disintegrate. This nuclear budding may represent the initial phase of nuclear fragmentation, which results in large nuclei becoming broken up into a number of smaller ones (Macklin¹⁶⁷). This is commonly seen in malignant cells *in vivo* (Howard and Schultz⁹⁸), and would seem to be directed towards increasing the area of the nuclear-cytoplasmic interface, which otherwise becomes progressively reduced, in proportion to the volume, as cells enlarge.

The disintegration of the nuclear structure may occur with or without the formation of acidophil deposits. In the latter case the chromatin particles coalesce and form irregular-shaped masses applied to the nuclear membrane (karyorrhexis), or it appears to liquefy (karyolysis). According to Roskin²⁰⁷, in degenerating tumour cells either chromatin accumulates so as to form hyperchromatinic nuclei, or else it is extruded into the cytoplasm and the nuclei become hypochromatinic.

Cell enlargement, with a relative increase in size of the nucleus,

frequently precedes nuclear disintegration. It occurs after irradiation in tumour cells (Mottram¹⁷⁵) and is followed by the disintegration of chromatin (thymonucleic acid protein complexes) with the formation of an achromatinic substance, which forms nucleolar-like bodies (Ludford¹⁴⁴). Mitchell^{172,173} has demonstrated that irradiation inhibits the synthesis of thymonucleic acid in the nucleus, and results in the accumulation of ribonucleic acid, mainly in the cytoplasm, and to a lesser extent in the nucleus. In the liver and kidney of vitally stained animals, and after poisoning with yperite and Lewisite, Wermel and Ignatjewa²³³ have described considerable enlargement of some cells. Their nuclei contained relatively large masses of nucleolar material. The formation of achromatinic substances within nuclei has received much attention in connexion with the intranuclear bodies, which are characteristic of some virus diseases, and will be further discussed in a later section.

Recent advances in microscopy have provided new techniques for investigating the structure of the nucleus of living cells. The phase-contrast microscope reveals the internal structure of the living nucleus with such clarity as to make it possible to study its modification under varying physiological and pathological conditions without having to resort to fixation and staining techniques. Another new field of investigation has been opened up by the demonstration by Ludford, Smiles, and Welch¹⁶¹ that the living nucleus can be studied by ultraviolet micrography employing wave-lengths of 2,570 Å and 2,750 Å without appreciable damage to the visible structures. By this means the resolution obtainable with the light microscope is doubled. Further, since nucleic acids exhibit their maximum selective absorption in the 2,600 Å region it becomes possible to study the distribution of nucleotides within the living nucleus under varying conditions of functional activity.

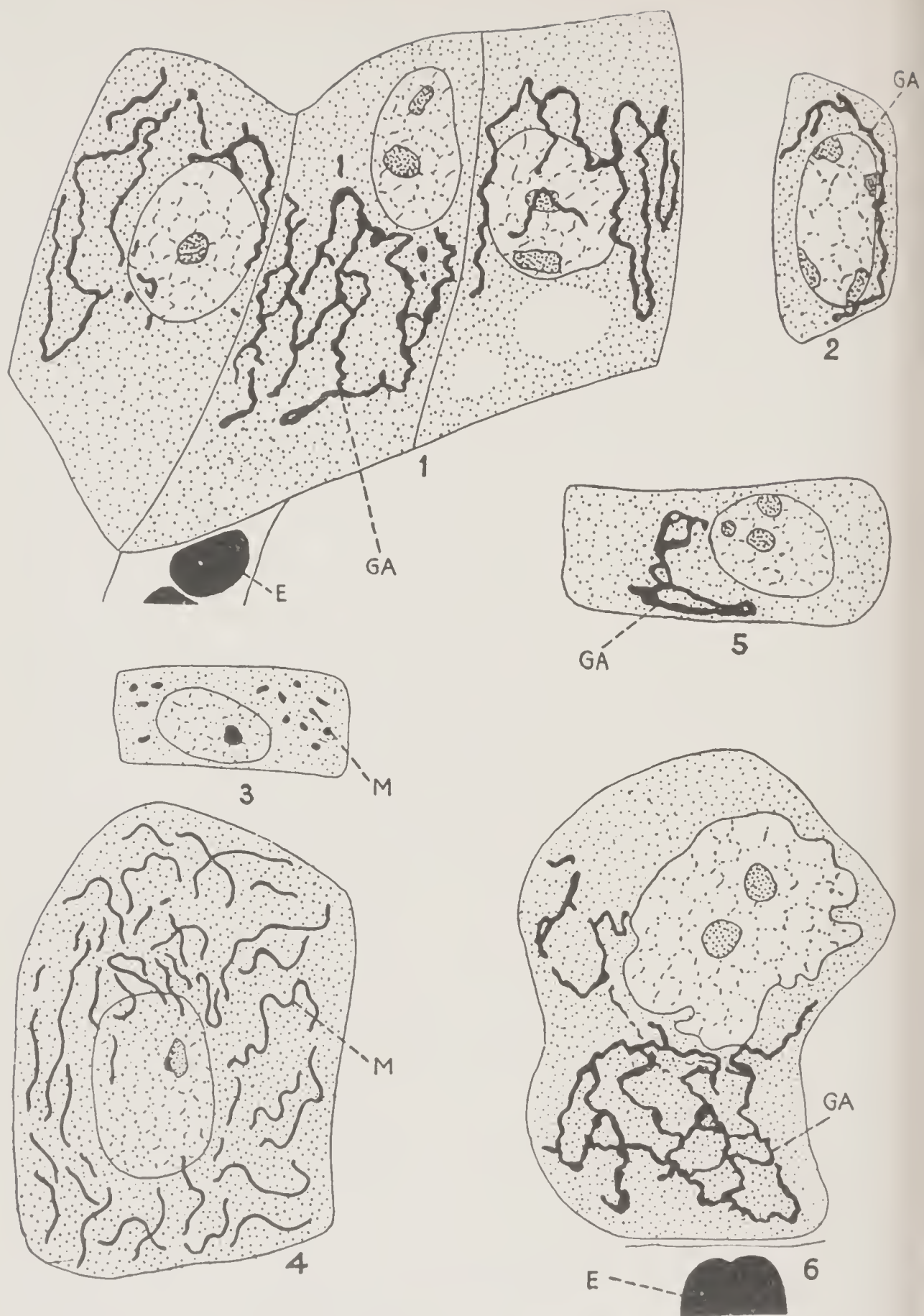
iii. CELLULAR HYPERACTIVITY

The cellular processes with which we have so far been concerned have been regressive in character. Tissue lesions, however, are invariably followed by intensified cellular activity directed towards the repair of injury or to functional compensation. Thus, destruction of muscle fibres by infiltrative tumour growth is often followed by growth of sarcoplasm at certain parts of the fibres, and a local increase in the number of nuclei. There is also an increase in the number of mitochondria and Golgi bodies (Ludford¹³³). With compensatory hypertrophy of the kidney, Hirsch⁹⁵ and others have described increased numbers of mitochondria. Nicholson¹⁸² observed the same in compensatory hypertrophy of the thyroid gland. In malignant glandular cells which retain their capacity to elaborate secretion, the mitochondria are numerous or elongated and the Golgi apparatus enlarged as in gland

cells engaged in the elaboration of their specific secretions. When tumours of this type are irradiated, there follows a period during which mitosis is inhibited, but the cells increase in size, and present the cytological characteristics of intensified secretory activity. The abortive acrosome formation induced by irradiation in spermatocytes of *Cavia* and *Abraxas* probably also results from a similar persistence of functional activity of the cytoplasmic organs during the period when the spermatocyte divisions are inhibited (Gatenby and Wigoder⁷⁷; Gatenby, Mukerji, and Wigoder⁷⁸).

One of the best examples of hyperactivity of cellular function is afforded by the thyroid gland in exophthalmic goitre (Graves's disease). Goetsch⁸¹ observed an increase in the number and length of the mitochondria in toxic adenomata of the thyroid associated with clinical symptoms of hyperthyroidism; and Bolt¹⁸ confirmed these observations. Cramer and Ludford⁵⁵ investigated the cytological changes in the thyroid gland under various physiological conditions, and found that the phase of intense secretory activity was characterized by long filamentous mitochondria and an enlarged Golgi apparatus. In a further investigation they studied the cytology of the thyroid in a case of exophthalmic goitre in the mouse, and three human cases of the disease (Ludford and Cramer¹⁵⁹). The most distinctive cytological feature was the extreme degree of enlargement of the Golgi apparatus. Text-fig. 2 shows a cell with the type of Golgi apparatus characteristic of the resting phase, while Text-fig. 1 represents three cells exhibiting the extreme degree of enlargement of the apparatus, from the mouse case. Mitochondria were numerous in the enlarged cells and were filamentous. In many of the cells they were rounded and swollen to form plastid-like bodies. Text-figs. 5 and 6 show the variation in size of the Golgi apparatus in the human cases, and Text-figs. 3 and 4 the difference in the mitochondria in a relatively inactive cell and in an actively secreting cell. It is of interest to observe that the human cases had been treated with iodine preliminarily to operative removal of the glands. In both the human and mouse glands reversal in polarity of the Golgi apparatus was observed (Text-figs. 1 and 6), so that it was suggested that this indicated the direct discharge of the specific hormone into the bloodstream.

Okkels¹⁸⁶ examined a large number of resected human thyroid glands. Forty-four of them were from patients with pronounced Graves's disease. These all exhibited enlargement of the Golgi apparatus. Another 14 cases were diagnosed histologically as colloid goitre with islands of epithelial hyperplasia. Ten of these were clinically thyrotoxic cases and showed enlargement of the Golgi apparatus; but of 39 simple colloid goitres, only 8 possessed a few scattered cell-clusters exhibiting enlargement of the Golgi apparatus. Twenty-five of Okkels's cases of Graves's



TEXT-FIGS. 1-6. Cellular hyperactivity in thyroid gland cells in exophthalmic goitre.

FIG. 1. Three enlarged cells showing hypertrophy of the Golgi apparatus, with reversal of polarity in the middle cell. FIG. 2. Relatively normal cell with type of Golgi apparatus characteristic of the 'resting' phase. FIG. 3. Mitochondria in an inactive cell. FIG. 4. Mitochondria in an actively secreting cell. FIG. 5. Golgi apparatus of cell in early stage of secretory activity. FIG. 6. Actively secreting cell with hypertrophied Golgi apparatus exhibiting reversed polarity.

E, erythrocyte in capillary; GA, Golgi apparatus; M, mitochondria.

Figs. 1 and 2, from a case of exophthalmic goitre in a mouse. Figs. 3-6, from human cases. After Ludford and Cramer¹⁵⁹.

disease had received iodine treatment, but although in many there resulted such an accumulation of colloid in the alveoli that the glands resembled simple colloid goitre, yet the Golgi apparatus of the glandular epithelium remained enlarged, indicating persistence of hypersecretion. Reversal of polarity of the Golgi apparatus was frequently observed. In one or two very grave cases of exophthalmic goitre, Okkels¹⁸⁶ reported that 'some alveoli were found in which the number of cells with reversed Golgi apparatus amounted to 126 out of a total of 146'. He does not consider that the position of the apparatus in the cell bears any relation to the direction of secretory discharge. His views, however, closely approximate to those expressed by Ludford and Cramer when he says that 'there is no cytological or histological reason to assume that in exophthalmic goitre the thyrotoxicosis is due to anything else than an excessive hypersecretion into the blood-stream, preventing the accumulation of colloid'. Gillman⁷⁹ constructed models of thyroid cells in various phases of functional activity, and since his models demonstrated that the Golgi apparatus became extensively spread around the nucleus during the phase of active secretion, concluded that reversal of the apparatus does not occur and is only seen in single sections. He agrees that there is considerable enlargement of the apparatus in exophthalmic goitre, and that in this disease it gives 'incontrovertible evidence of the marked activity of the thyroid cell'. He confirms Okkels's observation that 'iodine administration in exophthalmic goitre does not diminish the activity of the thyroid cell, which, instead, maintains its marked secretory powers: the products of this thyroid activity are stored in the follicles in the form of colloid' (Gillman⁸⁰). Thomas²²⁶, in his study of the human thyroid gland, distinguished between cells which elaborate secretion and others, the function of which is to excrete the hormone into the blood-stream. He confirmed the observations of Cowdry, Ludford, and Cramer and Okkels, that the Golgi apparatus does sometimes become displaced towards the basal end of the cells. This, he states, occurs after a period of intense secretory activity. He denies, however, that the position of the apparatus bears any relation to the direction of secretory discharge.

Welch and Broders²³¹ reported the results of a cytological examination of 73 thyroid glands removed at operations. Of 35 glands from patients with exophthalmic goitre, the Golgi apparatus was enlarged in all except one. This gland was removed from a patient who had been receiving iodine for 5 months before its removal. Further, in many of the follicles of glands removed from patients with exophthalmic goitre who had been treated with iodine, the cells showed marked regression, were flattened, and no enlargement of the Golgi apparatus was perceptible. Welch and Broders, therefore, do not agree with Okkels and Gillman as to the efficacy of iodine treatment as a means of inhibiting

hyperfunctioning of the thyroid cells. Of the other glands they examined they found enlargement of the apparatus in 19 of 21 glands removed from patients with adenomatous goitre with clinical symptoms of hyperthyroidism; and also in 5 of 16 adenomatous goitres from patients who were judged not to be suffering from hyperthyroidism. From a critical estimation of clinical, histological, and cytological observations they concluded that 'appraising the size of the Golgi apparatus in the thyroid gland would therefore seem to offer a more delicate method of estimating cellular and glandular function than ordinary methods'.

Researches of recent years have resulted in the discovery of chemical compounds capable of inducing hypothyroidism, and special attention has been devoted to the action of thiourea and its derivatives. In the thyroid cells of rats receiving large doses of thiourea over long periods Jones¹⁰¹ observed hyperplasia of the thyroid epithelium accompanied by depletion of colloid. There was considerable enlargement of the Golgi apparatus, but fragmented forms of the apparatus associated with vacuoles were not seen. As these are a characteristic feature of normally secreting cells, it was suggested that their absence was indicative of interference with the synthesis of thyroid hormone. Lever found that some of the thyroid cells of young cockerels, which were fed a diet rich in carbohydrate, with very little protein, exhibited reversal in polarity of the Golgi apparatus. When methyl-thiouracil was included in the diet about 60 per cent. of the thyroid cells exhibited reversed polarity of the Golgi apparatus. Lever¹¹⁰ suggests that interference with the intracellular secretion of the thyroid hormones results in a decrease of hormone in the blood, with a lowering of basal metabolism. This leads to an increased production of thyrotropic hormone by the hypophysis. He concludes that all cases of reversal in polarity of the Golgi apparatus can be explained as resulting from hyperactivity of the thyroid cells brought about by an excessive output of thyrotropic hormone.

iv. INTERACTION BETWEEN MICRO-ORGANISMS AND CELLS

a. The Action of Bacteria on Cells

Our knowledge of cytological reactions to bacterial infections is very incomplete. No detailed cytological investigation has been made by modern techniques of the reactions of the cells of the principal organs in the case of any bacterial or protozoan infection. Most bacteria grow intercellularly in the body fluids, giving rise to toxins which have a generally injurious action, while others exert their action more particularly on specific tissues. There are various references in the literature to bacteria and bacterial toxins inducing degenerative changes in cells. Thus Ciaccio³⁴ described the breaking-up and vesiculation of mito-

chondria in cells of the convoluted tubules of the kidney following injections of diphtheria toxin and cultures of *Escherichia coli*; and Ciaccio and Scaglione³⁵ reported similar changes in cells of the choroid plexus following injections of *Brucella melitensis* and diphtheria toxin, and also in phthisis. Choja^{32,33} studied the cytological changes in neurones of rabbits infected with certain viruses and some pathogenic bacteria and protozoa, and also poisoned by drugs such as strychnine, morphine, and nicotine. In general, the degenerative changes were of the same kind and involved reduction in the size of mitochondria, diminution in numbers, and finally their disappearance.

While the pathogenic bacteria are able to satisfy their nutritive requirements and grow in the body fluids, a few may also have an intracellular habitat. Thus *Neisseria intracellularis* and *Neisseria gonorrhoeae* occur in large numbers in the cytoplasm of polymorphs, in which they have been reported to proliferate. According to Goodpasture⁸² there is no evidence that the typhoid bacillus proliferates within the lumen of the gastro-intestinal tract, or within the blood-stream. He suggests that infection with this organism induces injury to the lymphoid tissue of the ileum, and results in the formation of plasma cells, which become infected. Within such cells in lymphoid follicles of iliac and mesenteric lesions he found small Gram-negative bacilli, which he regards as 'an especially small bacillary form of *E. typhi* modified by their intracellular environment'. They occur in clusters often surrounded by a capsular material, suggestive of active intracellular proliferation. It is suggested that newly formed plasma cells constitute the cellular hosts of the bacilli, affording nourishment and protection not only during the period of incubation but throughout the active course of the disease. Larger Gram-negative bacilli occur in the macrophages of the intestinal lesions, together with remains of phagocytosed lymphocytes, and in association with necrotic remains of macrophages, from which they derive nourishment.

The most intimate association between living cells and bacteria occurs with certain of the Mycobacteria. These are relatively large acid-fast bacteria, and include the causative organisms of tuberculosis and leprosy in man and animals. When the tubercle bacillus becomes lodged in the tissues it brings about an accumulation of cells around it, within which it is said to proliferate. Such collections of cells, known as epithelioid cells, constitute tubercles—the characteristic lesions of the disease. As they increase in size by the addition of fresh cells the centres undergo caseous degeneration, owing to defective nutrition and accumulation of waste products of metabolism. The origin of the cells forming the tubercles has been the subject of much discussion, but the application of the tissue-culture technique to this problem has done much to clarify it. Smyth²¹⁸ was the first to grow tissues *in vitro* with tubercle bacilli.

He employed chick embryo tissues, and observed the accumulation of lymphocytes around the bacilli, and the formation of epithelioid cells. Their nuclei increased in numbers, and as the result of cell fusion multinucleate giant cells originated. More detailed cytological studies were published later by Maximow^{169,170} and Timofejewsky and Benewolenskaja^{224,225}. Their investigations were carried out with rabbit tissues and blood leucocytes, and led to similar conclusions, namely, that epithelioid cells are transformed macrophages. Wermel²³² suggested they should be called 'epithelioid macrophages'. Morphologically, according to Lewis¹¹⁸, they are characterized by a central mass of fine granules stainable vitally with neutral red. This is surrounded by an intermediate zone of mitochondria amongst which fat droplets accumulate, especially in the cells at the centres of old tubercles. The oval nucleus lies excentrically in this zone, adjacent to the central granular area, and contains a more clearly defined nucleolus than that of mononuclears. Peripherally there is a clear hyaline layer often extending out into thin membranous pseudopodia, like those of macrophages. In tubercles, Lewis recognizes all stages between monocytes, macrophages, and epithelioid cells. He considers that monocytes which phagocytose relatively large particles, such as erythrocytes, become macrophages, while those that accumulate small granules become epithelioid cells. Macrophages acquire epithelioid characters after their phagocytosed particles have been digested to fine granules; and under certain conditions epithelioid cells, by ingesting large particles, become typical macrophages. For Lewis, therefore, monocytes, macrophages, and epithelioid cells represent the various functional variations of the same cell type. It is to be observed that lymphocytes have no place in this scheme, and herein lies his disagreement with Maximow, Timofejewsky, and Benewolenskaja, who consider that lymphocytes are capable of transforming into polyblasts, and thence to macrophages. Other workers, however, have been unable to satisfy themselves that lymphocytes behave in this manner (Jonescu and Jonescu¹⁰²).

Multinucleate giant cells of the Langhans type originate from epithelioid cells either by their fusion, or by nuclear without cytoplasmic division, or by a combination of the two processes. Both have been observed by Lewis^{119,120} in living cultures. These giant cells often attain considerable sizes with large numbers of nuclei. Lewis¹¹⁴ figures one with 47 nuclei. The cytoplasm of these cells exhibits the same structure as that of the epithelioid cells, and the nuclei are usually arranged either crescentically or circularly around the margin of the central granular area.

There has been considerable discussion as to the relation between the epithelioid and giant cells and the tubercle bacilli. Maximow^{169,170} and Haagen⁸⁹ spoke of symbiosis between rabbit epithelioid cells and tubercle

bacilli. Haagen reported that cultures of rabbit's lung with a moderate infection grow as well as non-infected controls. He described a chemio-tactic action on the part of the bacilli for macrophages, but was unable to come to any conclusion as to whether the bacilli after phagocytosis were able to divide inside the epithelioid cells. Maximow considered that the presence of bacteria occasionally exerted a stimulatory action on rabbit epithelioid cells, and Timofejewsky and Benewolenskaja refer to mitosis occurring in cells which were crammed full with bacteria. The Jonescus also reported mitosis in epithelioid cells containing degenerate bacilli. Since man is more sensitive to tubercle bacilli than rabbits, Timofejewsky and Benewolenskaja infected cultures of human buffy coat, and found that human leucocytes were far more sensitive to the action of the bacilli than were rabbit leucocytes. In heavily infected cultures the cells rapidly degenerated; and in slightly infected cultures in which tubercles were formed degeneration occurred in 4 or 5 days. Most of the papers concerned with tuberculosis *in vitro* refer to the destruction of bacilli by macrophages and epithelioid cells under certain conditions. According to Wermel²³², if the cells are able to overcome the infection the bacilli become surrounded by a vacuole, and are digested to a brown pigment. In his cultures of guinea-pig spleen he found that the bacilli had an inhibitory action upon growth; ultimately either the phagocytosed bacilli destroyed the cells, or else the cells digested the bacilli to pigment.

Although it has been said that 'tuberculosis is a disease of the monocytes', it is pertinent to note that masses of tubercle bacilli occur extracellularly in infected organisms; but since large numbers of bacilli occur in the cells of tubercles it is assumed that they find there a favourable habitat for their growth. The cellular changes occurring *in vitro* in the presence of tubercle bacilli, however, are by no means specific responses to infection with this organism. The formation of polyblasts, epithelioid cells, and multinucleate giant cells occurs regularly in cultures of lymphoid tissues. The same kind of cells are also formed by the monocytes of explanted tumours (Ludford¹⁴⁶).

The leprosy bacillus is still more adapted to an intracellular mode of life than is the tubercle bacillus, and the former is rarely found free in the tissues. It induces the formation of similar nodular lesions composed of the epithelioid type of cells and giant cells. These cells become densely packed with bacilli. From such a focus of infection cells infiltrate the surrounding tissues. Growth is by accretion of fresh macrophages, which become infected with the bacilli rather than by mitosis, which is rarely seen. Infection of the surrounding tissues appears to result from the breakdown of bacilli-laden cells. The result is that bacilli are found in many different kinds of tissue cells.

Benewolenskaja¹² investigated the reaction of cells to the leprosy

bacillus in tissue cultures. He employed cultures of normal and leukaemic human blood, and also cultures of liver, spleen, and lungs of human embryos. The bacilli had no harmful action on the cells, and in appearance the infected cultures scarcely differed from the controls. The macrophages which migrated from the explants were very active in phagocytosing the bacilli. After 5 to 7 days macrophages became transformed into 'lepra cells'. These cells, also called 'foam', or Virchow cells, are sometimes multinucleate, and contain many bacilli. Their cytoplasm presents a foamy appearance owing to the presence of numerous droplets of a lipoidal nature. They soon degenerated *in vitro*. By the fusion of macrophages or epithelioid cells there originated multinucleate giant cells. The phagocytosed bacilli continued to proliferate intracellularly, and the so-called 'cigar packs'—groups of bacilli resembling packets of cigars—were formed. Some of the phagocytosed organisms underwent degeneration, or were digested and gave rise to granules of yellow pigment.

Timofejewsky²²³ had previously grown *in vitro* fragments of leprous tissue from skin nodules of lepers. He observed the outgrowth of macrophages containing the bacilli, and fibroblasts devoid of them. He described the development of lepra cells, and the formation of minute lepra nodules composed of epithelioid cells derived from macrophages. Mitosis was occasionally seen in cells containing numerous bacilli. That infected cells are not seriously injured is indicated by the observations of Cowdry⁴⁷. He found that their nuclei and mitochondria were normal in appearance, however their functional activities were impaired. Their capacity to segregate trypan blue was reduced in rat leprosy, and they also failed to stain supravitaly with neutral red and Janus green. Cowdry also suggests that their nuclei contain relatively less thymonucleic acid than macrophages.

In the leprous lesions formed *in vivo* the bacilli form large globular masses called 'globi'. Cowdry⁴⁸ classifies them into three types: 'cigar packs', to which reference has already been made, are the smallest; 'seed globi', elongated bodies resembling oat seeds; and 'giant globi', which may attain a diameter of 150μ or more. Evidence has been adduced that the latter develop intracellularly in giant cells.

It is of interest to note that leprosy bacilli, which have become adapted to an intracellular mode of life, resist attempts to cultivate them by the routine methods of bacteriological technique. Benewolenskaja¹² reported their growth extracellularly in his degenerate cultures, and mentions that Timofejewsky's strain can be grown exclusively on clotted plasma, but grows much better in the presence of cells. It seems that the more micro-organisms become adapted to an intracellular habitat, the more difficult becomes their cultivation in artificial media. The *Rickettsiae* and viruses, to be discussed in the following sections,

have become so completely adapted to an intracellular mode of life that they are incapable of being cultivated apart from living cells.

b. The Action of Rickettsiae on Cells

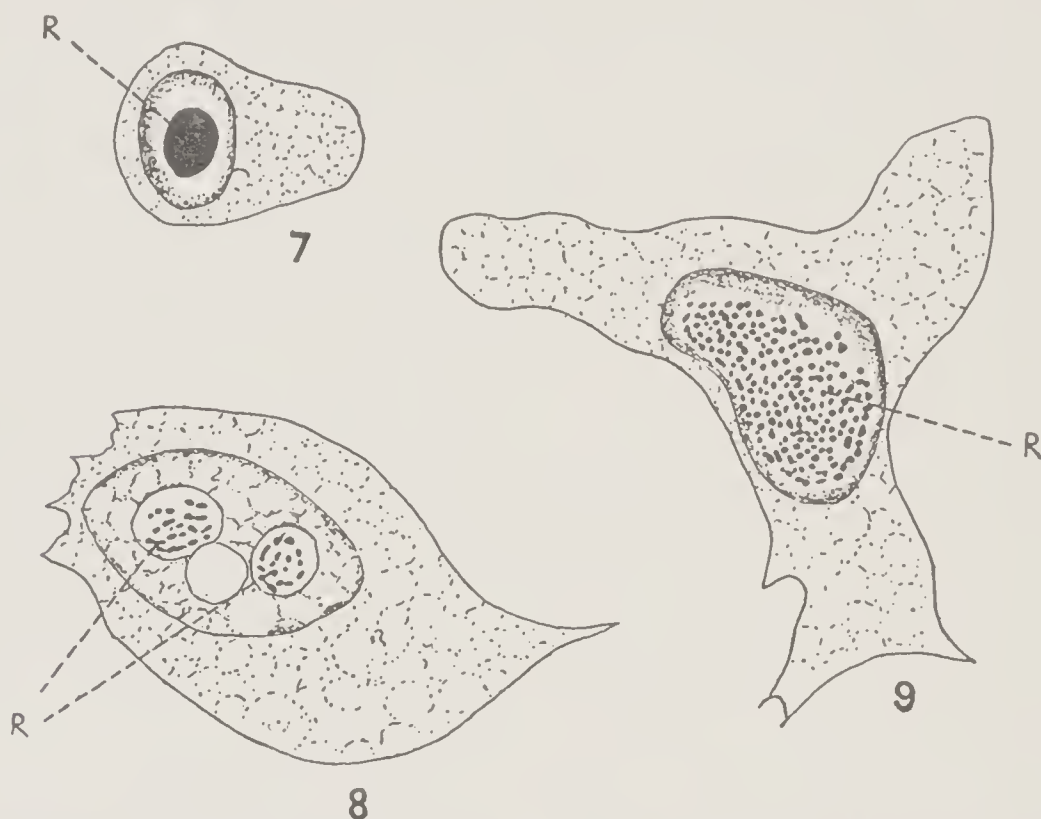
Rickettsiae have been defined by Cowdry⁴⁰ as 'Gram-negative, bacterium-like organisms of small size, usually less than half a micron in diameter, which are found intracellularly in arthropods, which may be more or less pleomorphic and stain rather lightly with anilin dyes, but which resemble in most of their properties the type species, *R. prowazeki*'—the causative agent of typhus fever. The generic name *Rickettsia* perpetuates the memory of Howard Taylor Ricketts, who died while investigating typhus fever; and the name of this species—*prowazeki*—was given in memory of von Prowazek, who also forfeited his life in the same cause.

Rickettsiae are transmitted to mammals by the bites of insects and arachnids. In the arthropod vectors they occur both intercellularly and intracellularly, but in their mammalian hosts are almost entirely intracellular. They occur most frequently in endothelial cells, but most species have also been reported in other cells. They are the aetiological agents of typhus fever, trench fever, Rocky Mountain spotted fever, heart-water of cattle, and Tsutsugamuchi disease. The more typical *Rickettsiae* have not been grown in artificial media apart from living cells, in which respect they resemble viruses, but they are larger than the elementary bodies of viruses, and although there is conflict of opinion as to their ultrafiltrability, most evidence indicates that they can be retained by filters which allow the passage of viruses.

Rickettsiae exhibit a high degree of adaptation to an intracellular habitat in the bodies of mammals. According to Wolbach, Todd, and Palfrey²³⁵, in man *Rickettsiae* tend to form intracellular globular masses. This feature is even more pronounced with *R. ruminantium* in cattle suffering from heart-water. Cowdry⁴¹ described colonies of the organism growing within vacuole-like cytoplasmic spaces filled with a clear fluid. The endothelial cells became considerably distended, but their nuclei seldom exhibited signs of injury. There was no evidence to indicate that the *Rickettsiae* stimulated cells to divide. Nicholson¹⁸² found that in cells infected with the *Rickettsiae* of Rocky Mountain spotted fever—*Dermacentroxenus rickettsi*—the mitochondria were reduced in numbers, and those remaining lost their rod-shaped and filamentous form and became granular. This condition, as has been previously described, is characteristic of early degenerative cellular changes.

Cytological studies of *Rickettsiae* in tissue cultures emphasize the dependence of these organisms upon living cells for their growth and survival. Pinkerton and Hass^{196,197} grew *in vitro* cells from the scrotal sac exudate of guinea-pigs infected with typhus. They obtained what

they considered to be relatively pure cultures of mesothelium. *Rickettsiae* grew abundantly in these cells, and they exhibited considerable morphological variations—minute granules, diplobacilli, and chain-like forms. The best growths of the micro-organisms were obtained at 32° C. The majority of the cells then became distended with the *Rickettsiae*. They were found in cells in all stages of mitosis. It is of interest to note that



TEXT-FIGS. 7-9. Intranuclear clusters of *Rickettsiae* of Rocky Mountain spotted fever in a tissue culture of scrotal sac exudate cells of the guinea-pig.

Fig. 7. Cluster of organisms with individuals not resolvable. Fig. 8. Early stage of infection. Fig. 9. Nucleus almost completely filled with *Rickettsiae*.

R, *Rickettsiae*. Semidiagrammatic, after Pinkerton and Hass¹⁹⁷.

40 per cent. of the cells in mitosis were free of *Rickettsiae*, while only 8.5 per cent. of the 'resting' cells contained no *Rickettsiae*. Mitosis was not sufficiently frequent to account for the infection of all the cells in the cultures. It seemed necessary to assume that the *Rickettsiae* were set free from some cells and gained entrance to others. The same mode of transmission was suggested by Nishibe, Hosono, and Myazawa¹⁸⁴ who worked with cultures of rabbit tissues infected with *R. orientalis* of Tsutsugamuchi disease. They observed no mitosis in infected cells.

In certain tissues of ticks Wolbach²³⁴ found *Dermacentor rickettsi* of Rocky Mountain spotted fever massed within nuclei. They have not been reported intranuclearly in infected cattle, but they have in tissue cultures of guinea-pig mesothelium. Pinkerton and Hass¹⁹⁷ were able to grow cultures of cells from the scrotal sac exudate of the guinea-pig infected with this *Rickettsia*. In several of their cultures the micro-organisms were seen only in the nuclei, while in others only the cytoplasm was infected. Both nuclear and cytoplasmic infection were rare.

The *Rickettsiae* made their appearance in the nuclei as small spherical groups of closely packed organisms. Each cluster was surrounded by a definite halo. 'In some instances the individual organisms of a group could not be resolved, and the clusters appeared as hyaline masses' (Text-fig. 7). These are of particular interest owing to their resemblance to the intranuclear bodies characteristic of certain virus diseases to be discussed in the next section. At later stages of infection the clusters within a single nucleus fuse together, and the mass thus formed may come to occupy the greater part of the nucleus (cf. Text-figs. 8 and 9).

Homogeneous inclusion bodies have also been described in the cytoplasm of cells infected with typhus *Rickettsiae*. According to Begg, Fulton, and van den Ende¹⁰ they probably only occur during the adaptation of a strain of *Rickettsiae* to a new host, and are indicative of cellular resistance to the infecting organism. *Rickettsiae* do not survive long in degenerating cultures. According to Pinkerton and Hass¹⁹⁷ the *Rickettsia prowazeki* disappears in less than a week, when the cells undergo degeneration. They remain visible only slightly longer than do the degenerating cytoplasm and nuclei of dying cells.

Recently Nyka¹⁸⁵ has brought forward evidence that in the lungs of rats infected with murine typhus, individual *Rickettsiae*, if not taken up by other cells, may develop extracellularly, giving rise to clusters and large masses of the organisms.

c. The Action of Viruses on Cells

(i) *The Nature of Viruses*. The study of viruses has opened up vistas of a world of minute organisms beyond the limits of resolution of the light microscope. Our knowledge of their existence results from the diseases they cause in animals and plants. These include such infections as small-pox, rabies, influenza, yellow fever, and poliomyelitis (infantile paralysis) in man; foot-and-mouth disease in cattle; and the mosaic diseases of plants. That there exists an as yet unexplored realm of sub-microscopic non-pathogenic organisms seems highly probable.

Viruses have only been cultivated in the presence of living cells. They are described as ultramicroscopic and filter-passing organisms. The former attribute refers to their size. While the *Bacillus prodigiosum*, one of the smallest of bacteria, colonies of which are responsible for 'blood-spots' on damp bread, has a diameter of 0.75μ , the Psittacosis virus, which is amongst the largest of the viruses, is only one-third that size. Of the smallest, yellow fever virus has a diameter of about 0.022μ , and the viruses of poliomyelitis and foot-and-mouth disease are approximately 0.01μ in diameter, which is smaller than some of the largest organic molecules. It is obvious that particles of such sizes cannot be resolved by the ordinary microscope. Viruses are described as filter-passing, since they can be forced through filters which retain the smallest

bacteria. Their actual sizes have been determined by three methods: by passage through Elford's collodion filters of known pore-size; by the technique of ultracentrifugation; and by direct measurement either of electron micrographs or, in the case of the larger viruses, of ultraviolet micrographs. The results obtained with the same virus by these different methods have been relatively uniform. The individual virus particles are usually known as 'elementary bodies'.

Many investigators have found it difficult to believe that the smallest of the viruses are living organisms. It seems scarcely feasible 'that there is room in a particle smaller than a molecule of haemocyanin and only two or three times the diameter of a haemoglobin molecule for the complexes necessary for organized life' (Laidlaw¹⁰⁷). Those who doubted the organismal nature of viruses found considerable support for their views in Stanley's discovery that from tobacco plants infected with mosaic disease there could be isolated a para-crystalline nucleoprotein which possessed all the properties of the virus (Stanley²¹⁹). Since infection of plants with this purified protein leads to production by the cells of more of the same virus, the suggestion has been advanced that viruses are complex auto-catalysts. Stanley suggests that 'the virus protein molecule may be able to cause smaller molecules already present in the cell, or smaller molecules produced by the cells as a result of the presence of the virus molecule to organize and combine to form a new virus molecule'. It is possible that all viruses are not of the same nature. Thus Rivers²⁰⁵ suggested 'some of them may be minute living organisms representing the midgets of the microbic world, others may be primitive forms of life unfamiliar to us, still others may be inanimate transmissible incitants of disease'. Laidlaw advanced the suggestion that the diminution in size of the various viruses may be correlated with progressive loss of ferment-systems and auto-synthetic potentialities, and an increasing degree of dependence upon their cellular host. According to this conception, 'viruses are decadent forms of organisms degraded through long persistent parasitism', the smallest having attained 'the supreme summit of parasitism'.

Cells infected with viruses react in various ways. Andrewes⁵ has emphasized that 'one can point to examples of every type of change produced in cells by the action of a virus, from pure necrosis to almost pure proliferation'. He cites, as instance of the former, foot-and-mouth disease. The filterable tumours of fowls afford examples of growth stimulation. With other viruses, such as those of fowl-pox and molluscum contagiosum of man, an initial cellular proliferation is followed by degeneration and necrosis.

(ii) *Virus Bodies*. Most viruses lead to the formation in infected cells of atypical bodies and granulations known as 'cell inclusions' or 'virus bodies'. They are formed either within the nucleus or in the cytoplasm.

and with some viruses, such as small-pox, both types occur. The nature of these virus bodies has been the subject of much discussion. With most of them there has been controversy as to whether they represent accumulations or colonies of the viruses, or are simply reaction products of the cells. There has been an unfortunate tendency to study infected cells cytologically, without a preliminary study of the morphological changes which occur in the same cells during autolysis, in the absence of micro-organisms. At the beginning of this article we described the regressive changes that occur in cells, and the illustrations on Plate 1 demonstrate the variety of granules of various kinds which originate in cells grown under aseptic conditions, some of which, owing to their size, might easily be confused with viruses.

It would be impossible here to survey at all adequately the vast literature devoted to the action of viruses on cells, so we shall restrict our attention to a few viruses that induce the formation of characteristic inclusions. These are enumerated below, together with their associated virus bodies.

Cytoplasmic inclusions

Fowl-pox	Bollinger bodies
Molluscum contagiosum of man	Molluscum bodies
Vaccinia (cow-pox)	Guarnieri-bodies
Rabies	Negri bodies
Mosaic disease of plants	Granular and crystalline inclusions

Intranuclear inclusions

Yellow fever	} Type A (Cowdry ¹⁶)
Salivary gland disease of guinea-pigs and moles	
Virus III infection of rabbits	
Herpes	
Poliomyelitis (infantile paralysis) (Covell ³⁷ ; Hurst ⁹⁸)	} Type B
Borna disease of horses (enzootic encephalomyelitis)	

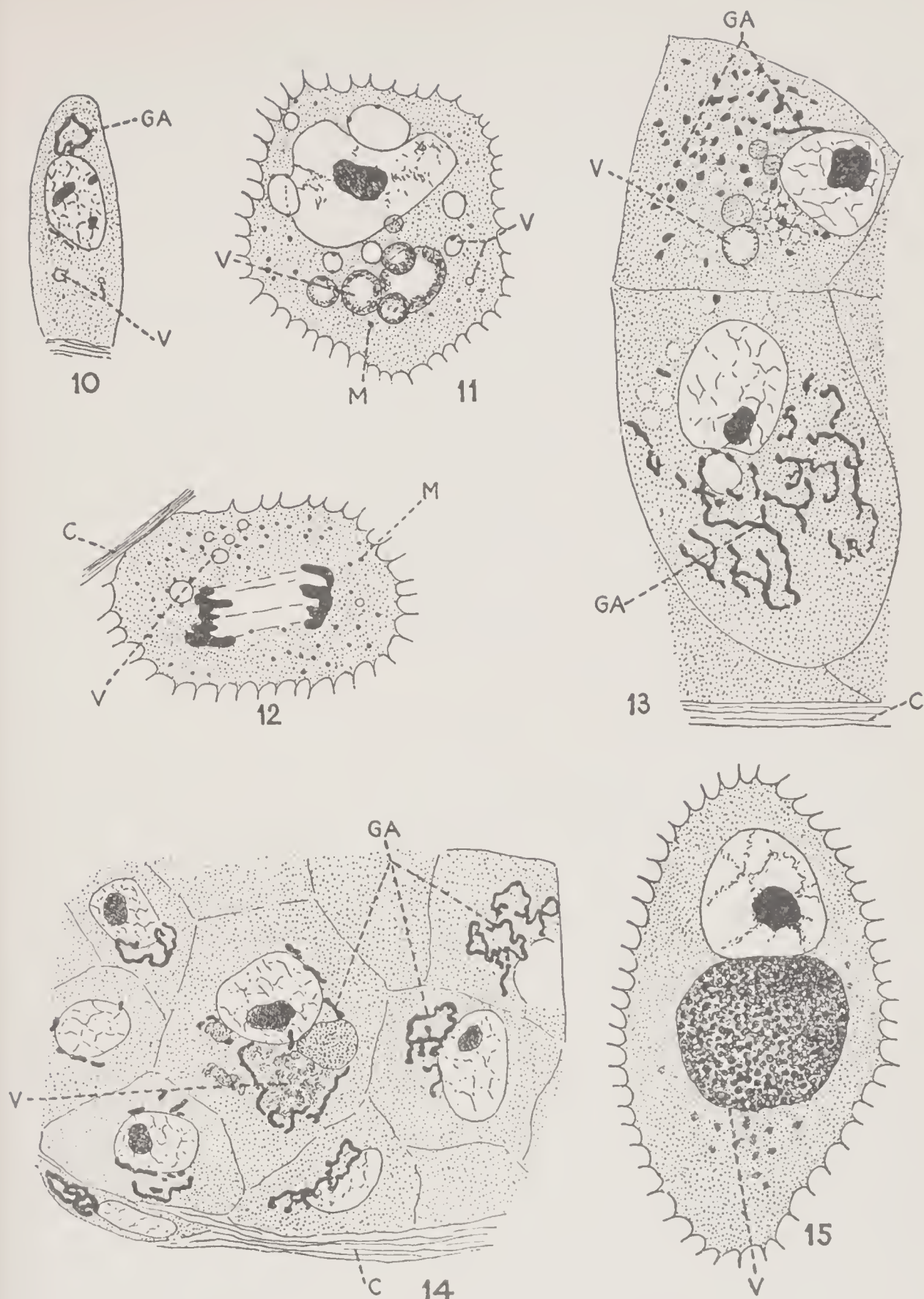
For full references to the literature the reader is referred to the reviews of Findlay and Ludford⁶⁸, Cowdry⁴², Rivers^{204,205}, Ludford¹⁴², and Stanley²¹⁹.

Much progress has already been made in the study of purified preparations of viruses by means of the electron microscope. Technical difficulties have so far restricted its range of usefulness for investigating the reactions of cells to virus infections. Considerable advances can be anticipated with improvement in the methods of preparing cells for examination by this new technique, with its hundredfold extension of the limits of microscopic resolution.

(iii) *Cytoplasmic Inclusions*. *Fowl-pox*. Scarification of the skin of fowls with this virus induces the formation of wart-like nodules, which

often attain a considerable size. As the disease is contagious it is sometimes known as epithelioma contagiosum. The earliest indication of infection in the epidermal cells is the appearance of small cytoplasmic vacuoles. Text-fig. 10 shows such a cell impregnated to demonstrate the Golgi apparatus, occupying here its usual position at the apical pole of the nucleus. Many cells are seen in division containing these small vacuoles. One is shown in Text-fig. 12. The mitochondria here are of the typical granular form characteristic of mitosis in epidermal cells. In examining sections of lesions, as one passes from the lowest layers of the epidermal cells towards the surface of the skin, the cytological features exhibited are the expression of two processes—keratinization, and progressive infection of the cells with the virus. Keratinization alone is accompanied by a gradual disintegration of all the cell organs (Ludford¹³⁰), and it has been suggested that the formation of the large inclusions in this disease is correlated with disintegration of the cellular organization. In the course of the intracellular infection the cytoplasmic vacuoles increase in numbers and in size (Text-fig. 11) and appear ultimately to fuse, so that most cells come to contain a single large inclusion—the Bollinger body—occupying most of the cell. During their enlargement the vacuoles become surrounded by a lipoidal substance which blackens with osmic acid, stains with Scharlach R. (Michaelis), and gives a positive reaction with Ciaccio's method for lipoids. After bleaching sections with hydrogen peroxide it is possible to demonstrate fine granules within the larger vacuoles, which have been regarded as the actual virus particles (elementary bodies), or accumulations of them (Text-fig. 15). During the early development of the virus bodies cells become hypertrophied and the Golgi apparatus considerably enlarged (Text-figs. 13 and 14). Its normal polarity is lost and in some cells is completely reversed. A close topographical relationship between it and the developing virus bodies is often clearly apparent, and it seems probable that it may play some role in the formation or localization of the lipoidal substance which is deposited around the developing inclusions. It breaks up some time before the latter are fully formed (Ludford and Findlay¹⁶⁰). A similar hypertrophy of the Golgi apparatus followed by disintegration was described by del Rio-Hortega⁵⁸ in nerve cells in a case of paralytic rabies, by Findlay^{66,67} in connective tissue cells in infectious myxomatosis of rabbits, and in some liver cells of animals infected with Rift Valley fever, and by Gresson and Zlotnik⁸⁶ in neurons of the central nervous system of sheep infected with louping ill.

Fowl-pox inclusions were subjected to micro-incineration by Danks⁵⁶. They left a greyish-white residue consisting of minute particles of mineral ash, corresponding to the granules seen in suitably stained sections (Text-fig. 15). He suggested that these granules might not



TEXT-FIGS. 10-15. Reaction of epidermal cells of the chick to the virus of fowl-pox. Fig. 10. Cell from lowest layer of stratum mucosum, showing earliest indication of infection—small vacuoles in the cytoplasm. Fig. 11. Cell with virus inclusions, the larger with peripheral granules. Fig. 12. Telophase at early stage of infection, showing virus vacuoles and mitochondria. Fig. 13. Two cells at early stage of infection. The lower is considerably enlarged and its Golgi apparatus is hypertrophied and reversed in polarity; in the upper cell the Golgi apparatus is fragmented. Fig. 14. Group of cells showing altered polarity of the Golgi apparatus, and the formation of virus bodies in intimate relationship with the Golgi apparatus. Fig. 15. Cell with a single Bollinger body exhibiting a finely granular structure. C, connective tissue of dermis; GA, Golgi apparatus; M, mitochondria; V, virus bodies. After Ludford and Findlay¹⁶⁰.

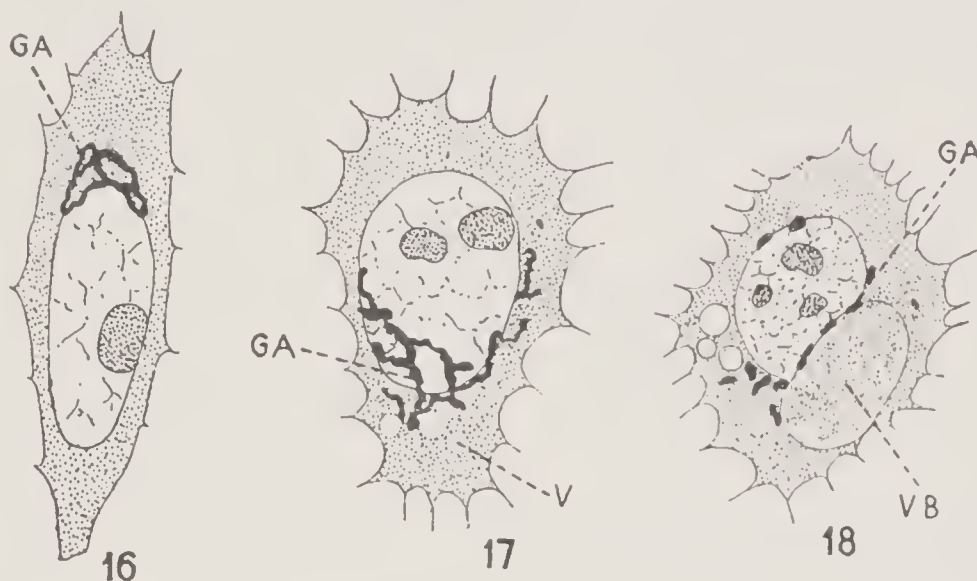
themselves be the virus particles, but that they served as loci for their adsorption. Woodruff and Goodpasture²³⁶ demonstrated conclusively that Bollinger bodies contained the virus by isolating single bodies by means of the micro-manipulation technique. One of these, after washing in saline and inoculation into the skin of a fowl, would produce a typical fowl-pox lesion, while the saline in which it was suspended before inoculation was innocuous.

Molluscum contagiosum of man. According to Goodpasture and King⁸³ molluscum bodies are very similar to the Bollinger bodies. They differ from the latter in the absence of a lipoidal envelope. After tryptic digestion they are sticky and gelatinous (Goodpasture and Woodruff⁸⁴). The fully formed body, according to Van Rooyen²²⁸, is pear-shaped, with a semi-translucent 'cap' at its conical extremity. This is the thinnest part of the body wall, and the most easily ruptured with liberation of the included granules. Van Rooyen considers this virus body resembles in miniature a fungus sporangium. Tests with Lugol's iodine indicate that its outer covering is composed of carbohydrate. After micro-incineration this is burnt off, leaving a granular residue derived from its contents (Van Rooyen²²⁹).

Vaccinia (Cow-pox). The Guarnieri-bodies produced by this virus have been the subject of much investigation, and most conflicting views have been expressed as to their nature. Since they are formed in epidermal cells in which keratinization occurs, with extrusion of nucleolar material into the cytoplasm, there has been some confusion of this with the virus inclusions. Most recent work favours the view that Guarnieri-bodies represent a developmental phase in the life-cycle of the virus. Bland and Robinow¹⁷, who observed the results of infecting cells in tissue cultures with the virus, came to the conclusion that the Guarnieri-bodies represent colonies of elementary bodies enveloped in a matrix.

In epidermal cells of the rat cornea infected with vaccinia, and treated cytologically for the demonstration of the Golgi apparatus, minute vacuoles are seen in the cytoplasm. The Golgi apparatus of such cells is frequently enlarged and often exhibits complete reversal of polarity. This condition is illustrated in Text-fig. 17, which should be compared with Text-fig. 16 showing the form and position of the apparatus in a normal cell. Other cells contain larger vacuoles that are seen to contain extremely fine granules, which are interpreted as accumulations of the elementary bodies. Such a cell is represented in Text-fig. 18. It will be observed that the Golgi apparatus is fragmented, and this therefore would indicate a later stage of infection (Ludford¹³⁷). The large vacuolar inclusions appear to be formed as the result of the enlargement and fusion of the smaller vacuoles. Ultimately cells become distended with large vacuoles filled with fine granules. That Guarnieri-bodies develop

from elementary bodies which penetrate cells, become surrounded by vacuoles inside of which they proliferate and finally fuse to form a virus colony included within a fluid matrix, was the conclusion to which Eisenberg-Merling⁶³ also came as the result of the study by dark-ground illumination of living infected cells of the rabbit cornea. It is of interest to note that the enlargement of the Golgi apparatus, its



TEXT-FIGS. 16-18. Reaction of rat corneal cells to vaccinia virus (cow-pox).

Fig. 16. Normal cell with Golgi apparatus at apical pole of nucleus. Fig. 17. Enlargement and reversal in polarity of the Golgi apparatus in association with virus vacuoles. Fig. 18. Fragmented Golgi apparatus and large Guarnieri-body.

GA, Golgi apparatus; V, virus vacuole; VB, virus body (Guarnieri-body). After Ludford¹³⁷.

reversal of polarity, and topographical relationship with the developing inclusions is closely paralleled with the cytological process described previously in cells infected with fowl-pox.

Psittacosis is another virus which forms cytoplasmic bodies of a well-defined character, composed of virus particles embedded in a matrix. They have been studied *in vivo* and *in vitro* by Bedson and Bland^{8,9} and Bland and Canti¹⁶.

Rabies. Two types of inclusions have been described in the nerve cells of animals and man infected with rabies: small hyaline granules, called 'lyssa bodies', and the larger Negri bodies, which consist of a ground substance containing a central body surrounded by chromatoid granules. The latter are sufficiently distinctive to be of diagnostic value. There is considerable difference of opinion as to whether they represent colonies of elementary bodies or are simply products of cellular disintegration. The latter view is supported by Covell and Danks³⁹.

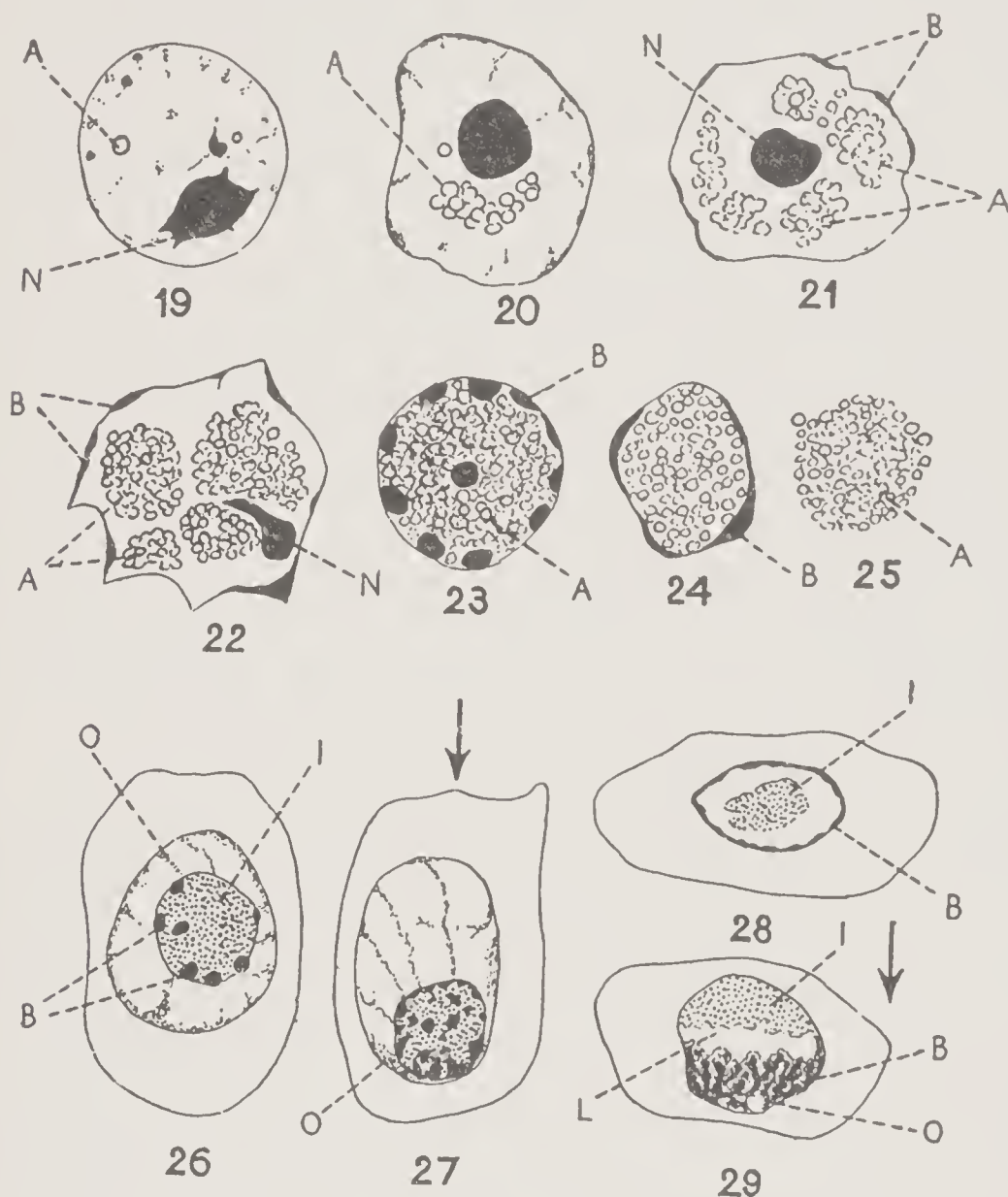
Virus Diseases of Plants. Cell inclusions are formed in some virus diseases of plants, but not in others. Two types have been described, amorphous granular bodies, and crystalline ones. Both contain virus together with a second constituent differing in the two types. Sheffield²¹⁵

has suggested that viruses may be rendered insoluble in the cytoplasm by precipitation in the form of such complexes, and that the formation of these inclusions may represent an intracellular protective mechanism.

(iv) *Intranuclear Inclusions*. Cowdry⁴⁶ distinguishes two types of intranuclear inclusions in virus diseases, which he designates types A and B. Those of type A are formed by the accumulation of granular acidophil material, accompanied by the displacement on to the nuclear membrane of the basophilic nuclear component. The nucleoli are altered and displaced peripherally, where they disintegrate, and the inclusion-laden nuclei ultimately become disrupted. Type B inclusions are usually spherical, often hyaline, droplet-like bodies, arising in nuclei which otherwise appear normal. Margination of the basophilic nuclear substance does not occur in the same way, but it is displaced peripherally if the inclusion becomes very large.

Luger and Lauda¹⁶³ interpreted these inclusions as arising from what they called 'oxychromatic' degeneration. The process of formation of a characteristic intranuclear inclusion is shown in Text-figs. 19-25, reproduced semidiagrammatically after Cowdry and Kitchen⁴⁹. They illustrate the formation of the inclusion bodies of yellow fever in the nuclei of liver cells of the monkey, *Macacus rhesus*, demonstrating stages in the progressive accumulation of acidophil granules, and the deposition of basophilic substance on the nuclear membrane. The intranuclear inclusions of liver cells in Rift Valley fever arise in a similar manner, according to Findlay⁶⁷. Intrahepatic inoculation of herpes virus into the liver of the monkey *Cebus hypoleucus* is followed by a different mode of formation of inclusion bodies. Cowdry and Kitchen⁴⁹ describe them as being formed from 'more or less even cloud-like deposits of acidophil particles in the centres of the affected nuclei'; also, the separation between the acidophilic and basophilic constituents of the nucleus is more marked in herpes than in yellow fever. Other differences have been described between the various intranuclear inclusions. Thus the herpetic inclusions are more finely granular and less compact than those of the virus III inclusions of rabbits (Cowdry⁴⁴). The development of the large inclusions within hypertrophied cells in submaxillary gland disease of guinea-pigs and moles is not accompanied by margination of basophilic material. The latter remains in association with the virus body and is responsible for its tendency to stain basophil. Lucas¹²⁶ has sought to correlate the degree of association between basichromatin and virus bodies with the cellular specificity of the viruses. 'When a virus is very selective, as it is in the submaxillary viruses, generally there is found a corresponding compatibility between inclusion body and the chromatin of the infected cell. In contrast, viruses having low specificity, such as herpes, which is cosmopolitan in infective poten-

tialities, show low compatibility with the nuclear material.' Lucas demonstrated this difference between the viruses he cites by subjecting cells containing the two types of virus bodies to ultracentrifugation.



TEXT-FIGS. 19-29. Intranuclear virus bodies.

Figs. 19-25. Yellow fever inclusions in liver cells of *Macacus rhesus* monkeys. (Semi-diagrammatic, after Cowdry and Kitchen¹⁹). Figs. 19-22. Stages in the development of the acidophil particles, and in the margination of its basichromatin. Figs. 23-4. Late terminal stage. Fig. 25. Nuclear membrane has disappeared, leaving a mass of acidophil granules. A, acidophil granules; B, basichromatin; N, nucleolus.

Figs. 26-9. After Lucas¹²⁶. Fig. 26. Duct cell of submaxillary gland of the ground mole containing intranuclear inclusion of maxillary gland disease. Fig. 27. Similar cell after ultracentrifugation for an hour at approximately 500,000 times gravity. Fig. 28. Corneal epithelial cell of rabbit containing virus body of herpes. Fig. 29. Similar cell after ultracentrifugation. B, basichromatin; I, inclusion body; L, nucleoplasm; O, oxychromatin.

The nucleus of an epithelial cell of the rabbit containing a central virus body and marginal basichromatin, as shown in Text-fig. 28, became stratified after centrifugation, as seen in Text-fig. 29. Basi- and oxychromatin were displaced centrifugally, the inclusion body centripetally. The virus body of a submaxillary gland cell of the mole (Text-fig. 26) was, on the other hand, displaced by centrifugation, together with the

basichromatin, in the direction of the centrifugal force, and strands of oxychromatin remained stretched between it and the nuclear membrane (Text-fig. 27).

The application of histochemical tests to the intracellular inclusions of yellow fever, herpes, chicken-pox, submaxillary disease, virus III, and Rift Valley fever have demonstrated that:

- (1) they do not stain with Sudan III or blacken with osmic acid;
- (2) they are negative to the Bensley-Macallum test for masked iron
- (3) they fail to stain with the Feulgen reaction for thymonucleic acid, except, in some cases, very faintly.

When subjected to micro-incineration the mature virus bodies, unlike nucleoli, leave very little or no ash (Cowdry⁴⁵; Cowdry and Kitchen⁴⁹; Findlay⁶⁷; Horning and Findlay⁹⁶; Rector and Rector²⁰²).

Acidophil intranuclear inclusions resembling those produced by viruses have been reported in cells under such conditions that there is no reason to assume any relationship with a virus infection—in kidney cells of many mammals and birds (Cowdry, Lucas, and Fox⁵⁰) and in tissue cultures of foetal leptomeninges (Fischmann and Russell⁷¹). Other inclusions have been found which are so like those of known virus aetiology that they have led their discoverers to assume they have been produced by unknown viruses. Examples of these are the inclusions found in liver cells of dogs, in salivary gland cells of *Cebus fatuellus*, and in kidney cells of *Macacus rhesus* by Cowdry and Scott^{52,53,54}; in kidney cells of sewer rats by Hindle⁹⁴; and the inclusions described by Hass⁹² in liver and adrenal cortex cells of an infant afflicted with a fatal disease characterized by hepato-adrenal necrosis. Intranuclear inclusion bodies resembling to varying degrees those produced by known viruses have been induced artificially in various nerve cells by injections of large quantities of distilled water and by hypertonic solutions of glucose, NaCl, and NaHCO₃; also in mononuclears and giant cells by certain aluminium and ferric compounds, and by carbon (Olitsky and Harford¹⁸⁸). In the latter case attempts to demonstrate a virus were unsuccessful. Furthermore, Belt¹¹ found intranuclear inclusions resembling those of yellow fever in liver cells in cases of liver necrosis following severe burns.

From the examples which have been cited it seems clear that the presence of intranuclear inclusions alone should not be accepted as proof of virus action. Whether any of the nuclear inclusions caused by viruses represent actual accumulations of virus particles is disputable. In justification of those who favour the view that certain of the inclusions consist of elementary bodies embedded in an acidophil matrix (Markham¹⁶⁸) it is pertinent to recall that intranuclear accumulations of *Derma-centroxenus rickettsi* occasionally appear identical with intranuclear virus bodies.

(v) *Viruses and Nucleic Acid Metabolism*. Nucleo-proteins have been found to be the main constituent of both plant and animal viruses. The plant viruses and the smaller animal viruses, e.g. poliomyelitis and louping ill, are composed of ribose-nucleotides. The larger animal viruses, such as vaccinia, molluscum contagiosum, and rabies, contain desoxyribose nucleic acid. Caspersson^{26,27} and his collaborators have shown that the building up of protein molecules by cells necessitates the presence of nucleic acids. Those of the desoxyribose type are necessary for the reproduction of the gene proteins. Oxyribose nucleic acid occurs in the nucleolus and in the cytoplasm, where it is concerned with the formation of the cytoplasmic proteins. Hydén¹⁰⁰ considers that viruses are to be regarded as 'self-reproducing units which act as parasites upon the nucleo-protein-forming parts of the host cells'. The various viruses attack different parts of the intracellular mechanisms for protein synthesis. Thus poliomyelitis and louping ill attack the heterochromatic parts of the nucleus. Rabies, neurovaccinia, and verruca attack the nucleolar stage in nucleo-protein formation, while the molluscum virus is parasitic upon the cytoplasmic protein-forming system. In each case the virus exploits some stage of nucleo-protein formation in the host cell in order to obtain material for its own growth.

V. CYTOLOGY OF CANCER

a. *Malignant Growth*

Malignant cells are characterized by their capacity for unlimited uncontrolled proliferation. Cells of any of the tissues of the body may become malignant and form a cellular mass, which invades the surrounding tissues. Small clusters of cells may be swept away in blood-vessels or lymphatics and give rise to secondary growths, or metastases, on becoming lodged in other organs. Thus cancerous growths of the skin of mice induced by painting with tar or carcinogenic hydrocarbons frequently metastasize in the axillary lymph nodes and lungs. A malignant growth originating in pigment cells of the human eye (melanotic sarcoma) may become so dispersed that most of the organs ultimately contain secondary growths, if the patient survives long enough.

Malignant growths derived from epithelial cells are known as carcinomata, and those arising from connective tissue cells as sarcomata. When grown as tissue cultures a typical carcinoma forms a sheet of closely adherent cells (Plate 2, Fig. 1), while a typical sarcoma tends to grow as a cluster of spindle-shaped cells (Plate 2, Fig. 2); but there are many exceptions to this generalization.

b. *Aetiology of Malignant Growths*

The results of experimental cancer research to date justify the classification of malignant growths into three classes, viz. (i) filterable tumours;

(ii) tumours in the aetiology of which a virus or virus-like agent is involved, and (iii) tumours originating in the absence of a demonstrable virus or virus-like agent. It must be emphasized that such a classification is a tentative one.

(i) *Filterable tumours*. A filterable tumour, on crushing to a pulp and mixing with saline, yields by Berkefeld-filtration a cell-free filtrate, which after injection into an animal of the same species reproduces the same type of progressively growing tumour as that from which the extract was prepared. Filterable tumours can also be transmitted by desiccated tissues, and by fragments of tumour preserved in 50 per cent. glycerine, as well as by cellular grafts.

Rous²⁰⁸ was the first to demonstrate that malignant growths could be transmitted by cell-free filtrates. He worked with five filterable sarcomata of fowls of different histological types, and subsequently many others have been discovered (Foulds⁷³). The potency of filtrates prepared from these tumours varies from time to time. Thus Gye and Andrewes⁸⁸ reported that for a period of 6 months the Rous sarcoma No. 1 failed to yield active filtrates, but did so spontaneously at the end of that period. The properties of the active agents contained in filtrates are essentially similar to those of viruses in general, so that they are usually known as tumour viruses. We are completely ignorant as to the means whereby these or other viruses stimulate cell proliferation. With regard to the tumour viruses two possibilities have been suggested. Either the virus alters a normal cell in some way so as to induce a kind of mutation and then merely proliferates as a passive passenger in the presence of the actively growing malignant cells, or the virus becomes intimately linked with the cellular mechanism so as to form some sort of cell-virus complex, which is the cause of malignant growth. Unequivocal evidence as to which is the correct explanation is lacking, but from what is known of other viruses and their relation to cells the latter seems the more feasible interpretation. Of all the known viruses, the tumour viruses exhibit the highest degree of cellular specificity and adaptation to their cellular hosts (Ludford¹⁵⁵).

Acidophil intranuclear inclusions similar to those characteristic of other virus diseases have been described by Lucké¹²⁸ in cells of a filterable tumour of the frog kidney, discovered by him. From his figures they appear to conform to the specifications of Cowdry's type A inclusions. Somewhat similar intranuclear inclusions have been described by Turevich²²⁷ in Fischer's filterable fowl sarcoma, which is indistinguishable from the Rous sarcoma No. 1. The cells of the latter tumour have been studied in tissue cultures by Tenenbaum and Doljanski²²². They observed the aggregation of oxyphilic granular material in the central part of nuclei suggestive of Cowdry's type A inclusions, and also intranuclear spherical bodies resembling his type B inclusions. There

was very wide fluctuation in the size of the cells. The smallest were approximately 5μ in diameter and the largest almost 500μ . The cytoplasm tended to undergo segregation into two clearly defined zones, a central body surrounded by peripheral protoplasm containing basophil granules, and thread-like structures. Frequently there were also cytoplasmic accumulations of a granular eosinophilic material. Altogether the cytological features were described as 'constituting a remarkable morbid picture, a distinctive syndrome of a severe cell disease'.

By electron microscopy, Claude, Porter, and Pickles³⁶ have demonstrated, in cells of two filterable fowl sarcomata, minute bodies of the same size as that estimated for the virus particles of these tumours by other methods ($700\text{ \AA}$ – $850\text{ \AA}$). Such particles were not found in normal fibroblasts or macrophages.

That the action of the virus in these cells is first to stimulate proliferation and then ultimately to induce progressive degenerative changes is confirmed by Tenenbaum's²²¹ work with tissue cultures of pure strains of the malignant cells. All attempts to maintain growth beyond 6 months failed. None of several strains of the tumour could be cultivated for more than 40 to 50 passages. However, Fischer⁶⁹ had previously grown the same tumour *in vitro* for $2\frac{1}{2}$ years, but he added fragments of tissue to his cultures from time to time, which presumably provided fresh fibroblasts for infection by the virus. Ludford¹⁵⁵ has shown, and Halberstaedter and Doljanski⁹¹ confirmed, that chick fibroblasts growing in tissue cultures can be infected with the virus of the Rous sarcoma which transforms them into malignant cells.

(ii) *Tumours in the aetiology of which a virus or virus-like agent is involved.* Shope²¹⁶ discovered that a virus was responsible for inducing wart-like growths—papillomata in cotton-tail rabbits. The virus could be transmitted to domestic rabbits, in which papillomata were formed that became malignant and gave rise to secondary growths in lymph nodes and lungs (Rous and Beard²⁰⁹). Later, Rous and Kidd²¹¹ showed that if tar was applied to the skin of rabbits so as to induce a local overgrowth of the epidermis (hyperplasia) and the Shope virus was then injected intravenously, malignant growths very soon developed at the site of the skin lesions. In the presence of methylcholanthrene papillomata became malignant within a few weeks (Rous and Friedewald^{74,210}). It was not possible to extract the virus from any of the carcinomata. Indirect evidence was, however, obtained, by means of serological and immunological tests, that the papilloma virus persisted in an altered form in the cells of two transplanted carcinomata that had originated in virus-induced papillomata. Most surprising are the recent findings of Smith, Kidd, and Rous²¹⁷, that having persisted in transplanted cancer cells 'for more than 5 years and throughout the first 25 successive tumor generations, the antigenic virus has now, after a further interval

of 5 years, disappeared from them; or at any rate it is no longer disclosed in them by serological experiments of the sort that regularly accomplished this before' (Kidd¹⁰⁴). The most obvious conclusion is that the maintenance of malignant growth is not dependent upon the continued presence of the virus, whatever part it might have played in the initiation of malignancy.

Considerable impetus was given to the search for viruses in mammalian cancers by Bittner's discovery that a virus-like agent was responsible for inducing breast cancer in high-cancer-strain mice. He showed by foster-nursing experiments (i.e. by transferring newly-born litters of high-cancer-strain mice to low-cancer-strain mothers, and the litters of the latter to high-cancer-strain mothers) that the incidence of breast cancer could be greatly reduced in high-cancer-strain mice and raised in those of the low-cancer-strain. The altered cancer incidence persisted in the progeny of the fostered mice (Bittner¹⁴). For the development of what Bittner called the 'inherited type of breast cancer' three factors were postulated: '(a) an active influence in the milk which is transferred to the young while nursing, (b) an inherited susceptibility, and (c) the growth of the mammary glands resulting from hormonal stimulation which will permit the cancerous change' (Bittner¹⁵). Further investigations showed that the 'active influence' was present in the spleen, thymus, lactating mammary tissue, blood, and breast tumours of cancerous strains. Recently Andervont⁴ has reported its presence in the seminal vesicles. The active agent was found to pass through Berkefeld filters, to be particulate, and to survive desiccation. It is referred to in the literature variously as the milk influence, milk virus, mammary-tumour agent, or breast cancer virus. Its action is limited to the induction of mammary cancer in mice. Certain strains of mice are very resistant to the agent, which they appear to inactivate.

The mammary-tumour agent of any one strain of mice induces breast cancers of varying histological types and rates of growth. The writer has grown many of these tumours in tissue cultures, and has been impressed by the similarity of the cells of the majority of them. By their nuclear characters it is not usually possible to distinguish them from mammary-gland cells grown from non-cancerous strains of mice. The polymorphism so often exhibited by cells of tumours induced by chemical carcinogens has not been observed. By electron microscopy, Porter and Thompson¹⁹⁹ have demonstrated in the cytoplasm of mammary-tumour cells of high-cancer-strain mice characteristic granules, with a central core approximately the same size as the virus-like particles observed in cells of the filterable fowl tumours (750 Å), although a less dense peripheral zone around each particle increases the average diameter to about 1,300 Å. Also, by employing the electron microscope, Passey, Dmochowski, Astbury, and Reed¹⁹² have demonstrated that extracts of

breast tumours and normal tissues of high-cancer-strain mice, as well as their milk, consistently contain spherical particles about 200–250 Å in diameter, which are absent from low-cancer-strain tissues and tumours of low-cancer strains, or at least are represented only by an occasional particle. Passey¹⁹¹ has pointed out that it has not yet been established if these particles are identical with the mammary-tumour agent.

The fundamental problem presented by the mammary-tumour agent is whether it persists in the cancer cells and is responsible for their malignancy, or whether it acts like a chemical carcinogen, bringing about some change in the intracellular mechanism, after which its presence is no longer necessary for the continuation of cancerous growth. The work carried out with the Shope papilloma virus has already demonstrated that a virus responsible for the induction of malignancy may persist for years in the cancer cells, apparently as a passive passenger, and then finally disappear without detriment to their proliferative capacity.

(iii) *Tumours in the aetiology of which no virus or virus-like agent has yet been discovered.* Bittner¹⁴ has pointed out that mammary cancer occasionally occurs in mice of low-breast-cancer strains, presumably without the participation of the mammary-tumour agent. Attempts to demonstrate its presence in tumours which arose spontaneously in low-cancer-strain mice have been unsuccessful (Ludford¹³³). Further, it could not be detected by the present experimental procedures in a mammary tumour which has been transplanted in hybrid mice for more than 40 years (Dmochowski¹⁹¹; Ludford¹⁵⁸). Then, strains of hybrid mice have been bred by Andervont⁴ with the specific aim of eliminating the mammary-tumour agent, yet cases of breast cancer still occurred. Also, a very high incidence of breast cancer has been observed by Orr¹⁹¹ in low-breast-cancer strains of mice treated with methylcholanthrene.

Andervont's⁴ experiments have demonstrated that there is 'a parallelism between an inherited tendency to mammary-tumor development and susceptibility to the mammary-tumor agent'. He adds that 'if those strains of inbred mice possessing an inherited predisposition to the development of mammary tumors are also susceptible to the mammary-tumor agent, then the role of the agent in the production of tumors could be that of an accelerator which markedly increases the incidence of tumors within the life span of the mice'. In this respect the agent would play a similar role to that of chemical carcinogens in the induction of lung tumours. Thus Heston⁹³ has reported the incidence of spontaneous lung tumours in a strain of Strong's A mice of 53 per cent. at 12 months, and Andervont³ has shown that lung tumours can be induced in practically all mice of this strain within 6 weeks by intravenous injections of dibenzanthracene.

Experimental cancer research has revealed a variety of means of inducing malignancy, which include cysticercus larvae aniline, tar,

numerous hydrocarbons, dyestuffs, ultraviolet radiations, radioactive substances, X-rays, and radium. Apart from their capacity to induce malignancy, these agents have one cytological action in common. They are all capable of bringing about aberrations of mitosis. Since ultraviolet and X-radiations, and certain carcinogenic hydrocarbons have also induced mutations in germ cells, it has been suggested that these, as well as the other carcinogenic agents, induce mutations in somatic cells, and that malignant cells are one of the types of mutations produced. The normal chromosome number is not uncommon in malignant cells, so it is postulated that cancer-producing mutations would be of the type involving changes in the distinctive gene material, which is probably of a protein nature. More recently it has been suggested that aberrant forms of plasmagenes (the hypothetical cytoplasmic genes) may be responsible for malignancy (Darlington⁵⁷; Haddow⁹⁰). Such conceptions of the mechanism of malignancy afford an explanation of the retention of specific cytological characters of different strains of transplantable tumours, which experience indicates may be retained indefinitely. Attention has frequently been drawn to the similarities between viruses and genes (Astbury⁶; Gowen⁸⁵), and it is not impossible that the intimate union of tumour viruses with cells might bring about disturbances of cellular metabolism similar to those induced by gene mutations.

While it is conceivable, but unproved, that all tumours might have a common virus aetiology, there is justification on general biological grounds for believing that the aberration of cellular functioning which is responsible for malignancy might be brought about either by the addition of an extrinsic agent or by some permanent alteration in the intracellular mechanism which is transmissible at mitosis. Many examples could be cited of the same cytological effect being induced by both biological and chemical agents. Normally, segmentation of the ovum is initiated by the sperm, but the numerous researches on parthenogenesis have demonstrated by what a variety of different methods the same result can be attained, even in the higher mammals. Specially significant in this respect are the similar cytological effects induced by viruses and chemicals. We have referred previously to the formation of acidophil intranuclear inclusions associated with virus diseases, such as those of yellow fever and herpes, and the production of identical bodies by Olitsky and Harford¹⁸⁸, when no virus could be detected. The inclusions produced by chemical agents gave the same histochemical reactions as those resulting from virus action.

c. Differences between Malignant and Embryonic Cells

Although no specific morphological criterion of malignant cells has yet been discovered, it is erroneous to conclude that there are no

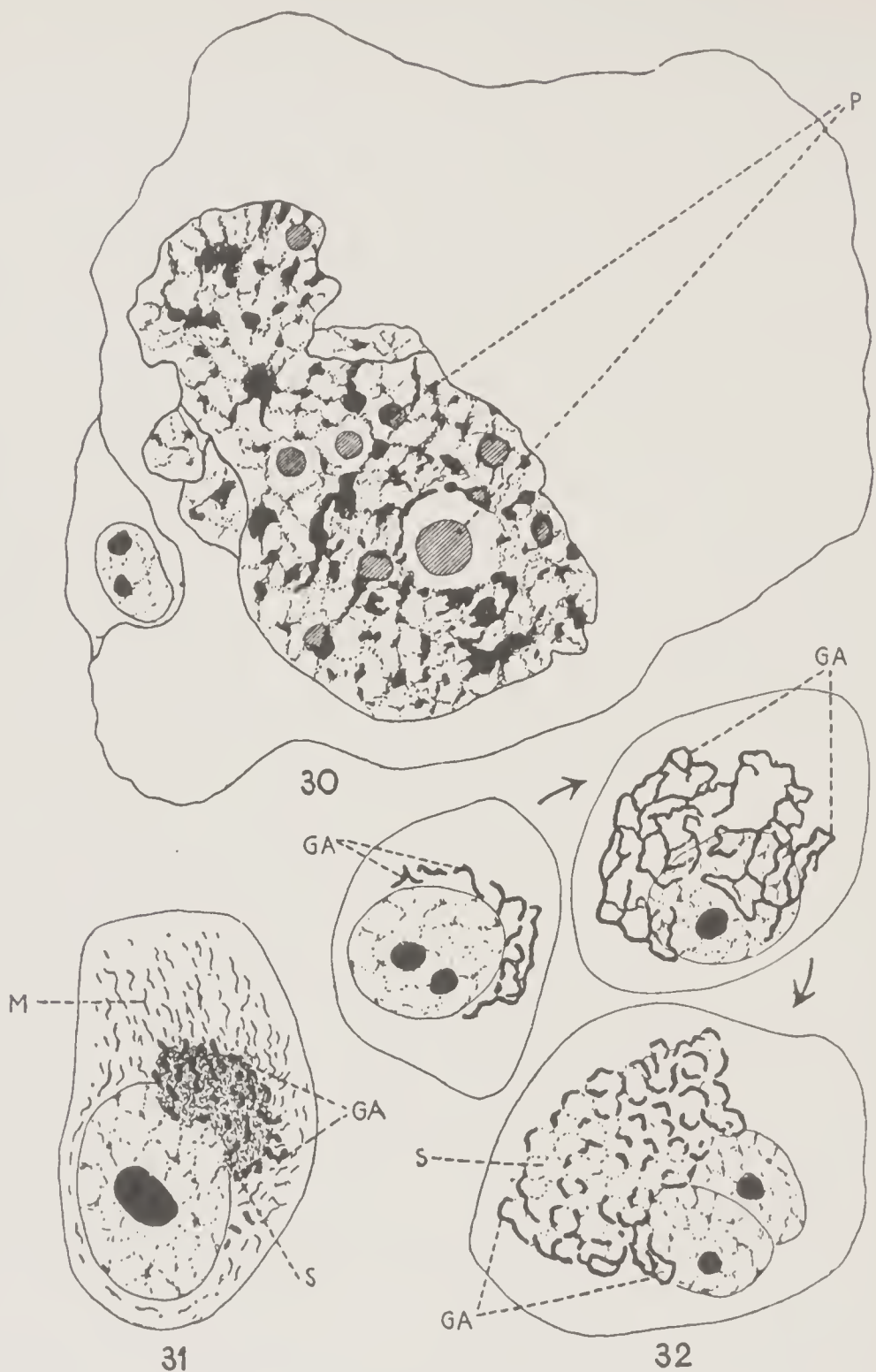
cytological differences between malignant cells and their non-malignant prototypes. Cancer cells are not simply cells which have acquired rapidity of growth. They are specifically altered cells. The transplantation of tumours has demonstrated that the specific cytological characters of tumours may be retained over prolonged periods of time. Some strains of transplantable tumours have been propagated for more than 40 years, and during that time retained unaltered their characteristic features—general rate of growth, degree of differentiation, and morphological structure. Further, these are retained just the same whether the tumours are propagated by transplantation in animals or grown as tissue cultures. The growth of normal and malignant tissues in cultures for long periods has demonstrated most definitely the essential difference between rapidly growing normal cells and malignant ones. Chick fibroblasts after cultivation *in vitro* for a period far in excess of the normal life-span of fowls cease to grow on inoculation into chicks (Carrel and Ebeling²²), while cultures of the Ehrlich mouse carcinoma, after twelve years' cultivation *in vitro*, when inoculated into mice continued to grow and form tumours of the same type as that from which they were originally explanted (Fischer and Davidsohn⁷⁰).

d. Variations in Structure and Functional Activity of Malignant Cells

Malignant cells often exhibit a high degree of dedifferentiation. In general, the more differentiated the cells the slower is their rate of growth. There are strains of transplantable tumours of the mammary gland the cells of which exhibit secretory activity, and others in which it has never been observed. Melanotic sarcoma cells elaborate pigment, and while some sarcomata induce considerable fibril formation, others do not. Malignant liver cells may excrete bile, and various tumours of the endocrine glands have been reported, the cells of which secrete to varying extents their specific hormones. Such cases render it improbable that cellular dedifferentiation can be an essential factor in carcinogenesis, as some have maintained.

Malignant cells exhibit the widest range of morphological abnormalities. Amongst cells of the same tumour there is frequently a considerable variation in size, as is shown in Text-fig. 30, which represents two cells from a melanotic sarcoma of the horse. Enlargement of cells is accompanied by increase in number of mitochondria, and the Golgi bodies become larger and more numerous. The polarized disposition of the cytoplasmic organs characteristic of so many normal cells is usually retained by malignant cells adjacent to well-formed stromata, but in the absence of the latter polarity is invariably lost (Ludford^{131,133}).

The structure of the Golgi apparatus of malignant cells corresponds to that of their prototypes or to stages in their dedifferentiation (Ludford¹³⁴). The specific secretions of malignant gland cells are formed in



TEXT-FIGS. 30-2. Variation in size, and examples of functional activity in malignant cells.

Fig. 30. Small cell and a greatly hypertrophied cell from a melanotic sarcoma of the horse. P, intranuclear pigment granules. After Ludford¹²⁹. Fig. 31. Mouse mammary carcinoma cell, showing relation between Golgi bodies and secretion. After Ludford¹⁴⁴. Fig. 32. Human mammary carcinoma cells, showing relation between Golgi bodies and milk.

Original. GA, Golgi apparatus; M, mitochondria; S, secretion.

relationship with the Golgi apparatus in the same manner as has been described in most glands. Text-fig. 32 illustrates the formation of milk in human mammary carcinoma cells, and Text-fig. 31 abortive secretory activity in a mouse mammary carcinoma.

Areas of necrosis are common in tumours and seem to result from

deficiencies in their blood-supply. Consequently, the cells of tumours exhibit all kinds of degenerative changes. Hypertrophied nuclei may become extensively lobed, and nuclear budding and fragmentation are common. Cell fusion (plasmogamy) results in syncytial masses being formed. Nuclear and cytoplasmic organs exhibit the regressive changes which were described at the beginning of this chapter.

A new impetus has been given to the study of living malignant cells by the advent of the phase-contrast microscope. The great advantage of this technique is that it enables both nuclear and cytoplasmic structures to be studied at the same time in the living condition. The figure of Plate 3 depicts a cell of a rapidly growing mouse sarcoma. The nucleus is lobed and contains several nucleoli with 'nucleolar-associated chromatin' (Caspersson²⁷), as well as numerous other particles of chromatin, which are stainable by the Feulgen technique. Amongst the many mitochondria and other granules accumulated at the side of the nucleus can be distinguished a canalicular system which corresponds to the Golgi network as demonstrable by osmic and silver-impregnation techniques. Cytological studies of living malignant cells by phase-contrast microscopy which have been made by Albertini^{1,2}, Zollinger^{238,239}, and Ludford, Smiles, and Welch¹⁶² confirm for the most part the validity of the older work carried out by the classical techniques of fixation and staining.

e. Mitotic Abnormalities of Malignant Cells

Since the early studies of Pianese and von Hanseemann the mitotic aberrations of malignant cells have received considerable attention. Although most of the abnormalities which have been reported in malignant cells have been produced experimentally in non-malignant cells, yet the occurrence of so many in cancerous tissues is a distinctive feature (Ludford^{140,141}). An experienced cytologist would have no difficulty in distinguishing between a tissue culture of actively growing embryonic cells and one of malignant cells on this basis alone. Almost all the types of mitotic abnormalities conceivable are met with in malignant cells (Plate 4). There is great variation in the numbers of the chromosomes, and the latter vary in shape from small coccoid bodies to long filaments; sometimes they are fragmented. The spindle is often multipolar, but occasionally absent altogether (Ludford¹³⁹). The chromosomes may fail to form a complete metaphase plate, and often some remain apart without attachment to the spindle. Unequal distribution of chromosomes to daughter-cells results from delayed separation of chromatids, or failure of some to separate at all. Then nuclear division is not always followed by cytoplasmic division. The occurrence of abnormal mitoses is more frequent in some tumours than in others. The fact that the abnormalities are retained during growth

in vitro implies that they are an inherent property of malignant cells and not the result of the actions of toxic metabolites accumulated *in vivo* owing to the deficient vascularity of tumours, as was at one time suggested. It would be extremely interesting to know whether any malignant growths occur without atypical mitoses of any kind.

The mitotic aberrations of malignant cells have been attributed to alterations in the colloidal state of the protoplasm of malignant cells (Ludford¹⁵⁴; Powell²⁰⁰), and to a quantitative change in their nucleic acid synthesis (Koller¹⁰⁶). Bieseke¹³ has drawn attention to the widespread occurrence in tumours of polytene chromosomes. They are believed to result from the centromere lagging behind the chromonema in growth and division during endomitosis. The larger volume of nucleolar material in cancer cells is presumably the outcome of an increased number of nucleolar organizers owing to the polytene condition of the chromosomes.

f. Differences in the Cytoplasm of Normal and Malignant Cells

That the cytoplasm of sarcoma cells differs morphologically from that of fibroblasts has been reported by Lewis and Gey¹²³, Lewis¹²¹, Carrel and Ebeling^{24,25}, and Ludford¹⁵⁰. A comprehensive study of the living cells of numerous sarcomata was made by Lewis¹²². He says:

Each type of malignant cell usually has one or more characteristics which differentiate them from normal ones. Some of the common differences which distinguish the malignant cells are increase in size of cell and nucleus, increase in density of cytoplasm, increase in number and decrease in size of the mitochondria, increase in the amount of nucleolar material, increase in thickness of the nuclear membrane and the granular condition of the nucleoplasm.

Differences between the cytoplasm and nucleoplasm of fibroblasts and sarcoma cells are clearly seen by examining living cells by dark-ground illumination. These are illustrated in Plate 5, which shows a typical fibroblast (Fig. 1) and cells of the Jensen rat sarcoma (Figs. 2 and 4), and of the Crocker sarcoma (Fig. 3). The more granular nature of the cytoplasm of the sarcoma cells is obvious.

Although Chambers and Ludford^{29,30} were unable to detect any differences in the viscosity of normal and malignant cells by the micro-surgical technique, or in the intracellular hydrogen ion concentration by the injection of colour indicators, the behaviour of tumour cells *in vitro* suggests that the colloidal state of their cytoplasm differs in some way from that of normal cells (Ludford^{148,149}). Fibroblasts are able to grow and spread out on a glass surface in a fluid culture medium such as serum. Many strains of tumours are quite unable to do so under normal conditions of growth *in vitro*; but there is much variation in the behaviour of the cells of different strains of tumours in this respect (Ludford¹⁵⁶). The rounding-up of cells of mouse sarcoma No. 37 in

serum, and their spreading-out in a plasma clot is illustrated in Plate 6. Fig. 1 is enlarged one-and-a-half times more than Fig. 2. Similar differences in behaviour have been reported between epithelial cells and carcinoma cells.

Probably related to a difference in the ultrastructure of their cytoplasm is the inability of malignant cells to segregate acid vital dyestuffs such as trypan red and trypan blue in the same manner as their normal prototypes. Fibroblasts segregate these dyes as granules in association with vacuoles. No sarcoma has yet been found the cells of which segregate these dyes *in vivo* as do fibroblasts (Ludford¹³⁸; Foulds⁷²). This is the more remarkable since Duran-Reynals⁶¹ has pointed out that an abnormal amount of dyestuff collects in tumours *in vivo*. In tissue cultures, probably owing to the altered condition of cells spread out in plasma clots, some segregation of acid vital dyes by sarcoma cells occasionally occurs; but there is a clear difference in the manner in which the dyestuff is segregated by such cells and normal fibroblasts. While milk in secreting mammary-gland cells is stained by trypan blue, and the same cells when not engaged in secretory activity segregate the dyestuff in granular form, malignant mammary-gland cells take up very little or no dyestuff, whether they be of the differentiated secretory type or have lost all secretory capacity (Ludford¹⁴⁵).

It is not known whether the failure of malignant cells to segregate acid vital dyes like their normal prototypes is the result of some difference in the ultrastructure of their cytoplasm preventing the actual segregation of the dyestuff in granular form from its semi-colloidal solution, or whether it results from a less permeable plasma membrane. Lipoid soluble basic dyestuff and coloured compounds of the Sudan III type readily penetrate living malignant cells and, in the case of the former, colour cytoplasmic vacuoles, while the latter stain fat droplets (Ludford¹⁵¹); but these are cases of the staining of preformed structures and differ essentially from the segregation of dyestuffs as granules which are new cytoplasmic formations containing a protein constituent (Chlopin³¹). Further investigations on the colloidal state of the protoplasm of malignant cells are required to elucidate the significance of the observations which have been recorded.

g. Nucleotides of Malignant Cells

Caspersson's^{26,27} work has led him to the conclusion that the cell nucleus is the centre of protein metabolism, and that nucleic acids are essential for all intracellular protein synthesis. In malignant cells, according to Caspersson and Santesson²⁸ the heterochromatin system is stimulated to abnormal activity. This results in disturbances in the formation of cytoplasmic proteins and in the reproduction of the gene proteins, and so leads to abnormal growth. It is suggested that carcino-

genic agents act by stimulating the heterochromatin system of groups of cells. The most actively growing malignant cells in the best vascularized parts of tumours are characterized by a normal-sized nucleus, particularly rich in desoxyribose nucleotides, and have large nucleoli. The ratio of nuclear to cytoplasmic volumes is above the average, and the cytoplasm contains large quantities of ribose nucleotides. There are transitional stages between such cells and others with larger nuclei poor in desoxyribose nucleotides, but with larger nucleoli containing considerable amounts of ribose nucleotides, which are absent from their cytoplasm. Poor nutrition is considered to be responsible for bringing about this cellular condition.

Caspersson's and Santesson's studies of malignant cells were based on ultraviolet micrographs of tumour tissue, which had either been fixed in formalin or prepared according to the Gersh freezing-drying method. Recently Ludford, Smiles, and Welch¹⁶² have made a comparative study of living malignant cells by phase-contrast and ultraviolet microscopy. They have demonstrated that it is possible to obtain ultraviolet micrographs of living cancer cells by employing objectives computed for working with wave-lengths of 2,570 Å and 2,750 Å, without appreciable injury to cell structures. With these wave-lengths micrographs of a rapidly growing sarcoma showed intense absorption by nuclear structures and by the cytoplasm. Mitochondria were specially conspicuous. They appear as strongly absorbing filaments in the cell depicted in Plate 7. The study of mitochondrial behaviour during all phases of growth suggests that they play an important role in cytoplasmic protein synthesis. Micrographs of living malignant cells fail to support Caspersson's ideas on the function of the 'nucleolar associated chromatin'. So many dividing cells at late stages of cleavage, before the reconstitution of nucleoli, exhibit considerable cytoplasmic absorption (Plate 8), and cells with well-defined nucleoli with associated chromatin granules have not revealed a more intense cytoplasmic absorption immediately around the nuclear membrane. On the contrary, the nucleus is often surrounded by a relatively clear area.

Comparison of micrographs of fibroblasts from connective tissues with malignant fibroblasts of fast and slow growing sarcomata has revealed a close correlation between the nucleotide content of the cells and their rate of growth. Particularly striking is the intense cytoplasmic absorption of rapidly growing sarcoma cells compared with the relatively clear cytoplasm of 'resting' fibroblasts.

The possibility of studying living cells by ultraviolet microscopy has opened up a new field for cytological investigation. Specially interesting is the observation that under appropriate conditions chromosomes can be distinguished in 'resting' nuclei as attenuated double threads upon which are disposed parallel pairs of minute granules which are not

properly resolved with a wave-length of 2,570 Å. From their size and mode of distribution, these granules would seem to represent genes. Thus in this technique we have the means for investigating the distribution of nucleotides inside the living nucleus, as well as in the cytoplasm, under varying conditions of growth and functional activity.

CONCLUSION

In the preceding pages we have discussed in a necessarily superficial manner some of the more important of the pathological aspects of cytology. The attempt has been made to give some idea as to how cells react to adverse conditions, to demonstrate the cellular mechanism tuned to its highest pitch in functional hyperactivity, and to trace the interaction between micro-organisms and cells which, on the one hand, results in cell destruction, and on the other, as the result of an increasing dependence of the micro-organism upon its cellular host, leads to a progressive perfecting of a symbiotic relationship, favouring the survival of both at the expense of the organism as a whole. The climax is reached with the filterable tumours where the union between virus and cell is so complete as to confer upon the cell new morphological characters and remarkable proliferative capacity. The cause of the unlimited uncontrolled growth of the majority of tumours still remains the subject of acute controversy and unremitting research.

The problems encountered in this survey have been many and varied, and there are numerous and extensive gaps in our knowledge, so that at the present time a synthetic discussion seems unprofitable. We are left with the conception of the cell as a functioning unit, with its microscopically resolvable organelle reacting to abnormal conditions as part of a unified system. Only the grossest parts of the mechanism are visible under the light microscope, which conveys but the crudest idea of the underlying ultramicroscopic complexity. The advances in microscopy of recent years, especially phase-contrast and ultraviolet microscopy, and the advent of the electron microscope, have provided new and potent instruments of research. By their skilful application we may reasonably hope to acquire a more fundamental insight into the mechanism of cellular functioning under normal and pathological conditions.

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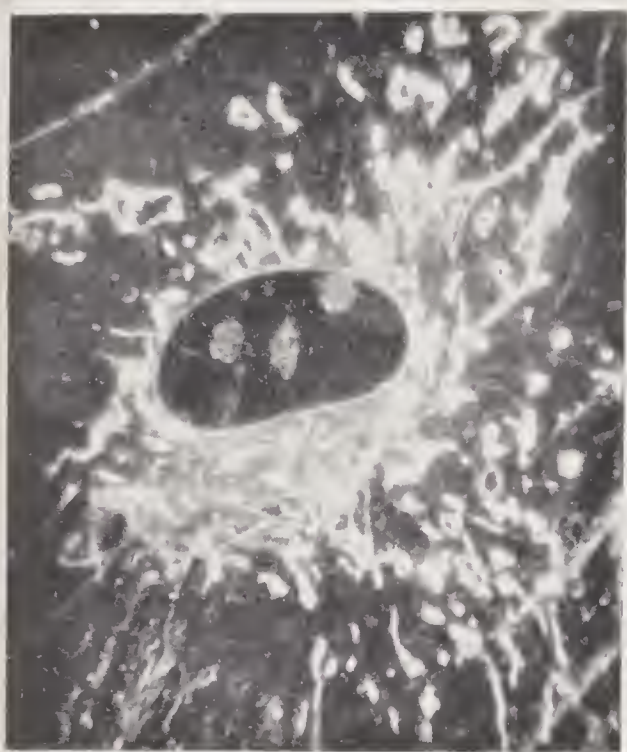


FIG. 1. Accumulation of degenerating mitochondria around the nucleus

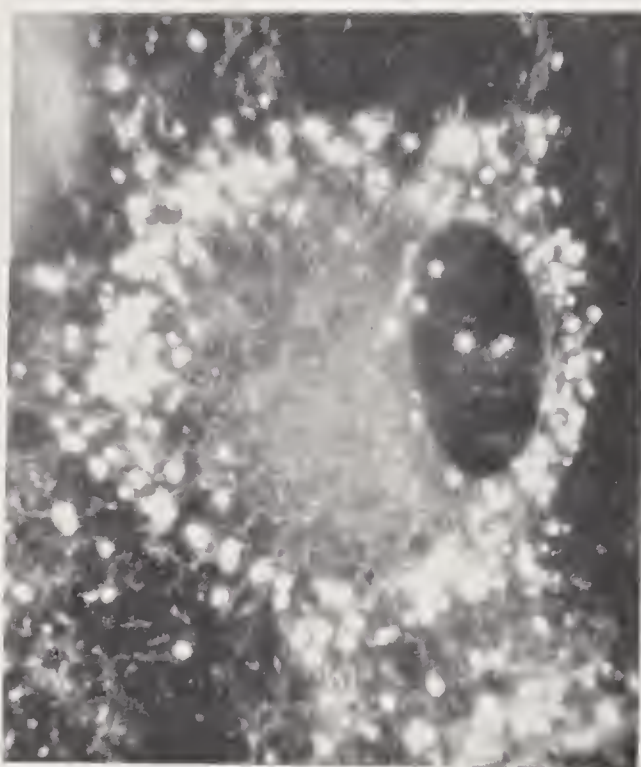


FIG. 2. Ill-defined mitochondria and degeneration granules accumulated at the side of the nucleus, and surrounded by fat droplets



FIG. 3. Vacuolation of the cytoplasm
(Original)



FIG. 4. Binucleate cell filled with degeneration granules in the midst of which is a reticulate space resembling the Golgi apparatus

Degenerative changes in fibroblasts in living-tissue cultures

Figs. 1, 2, and 4 after *Ludford*¹⁵³

R. J. Ludford

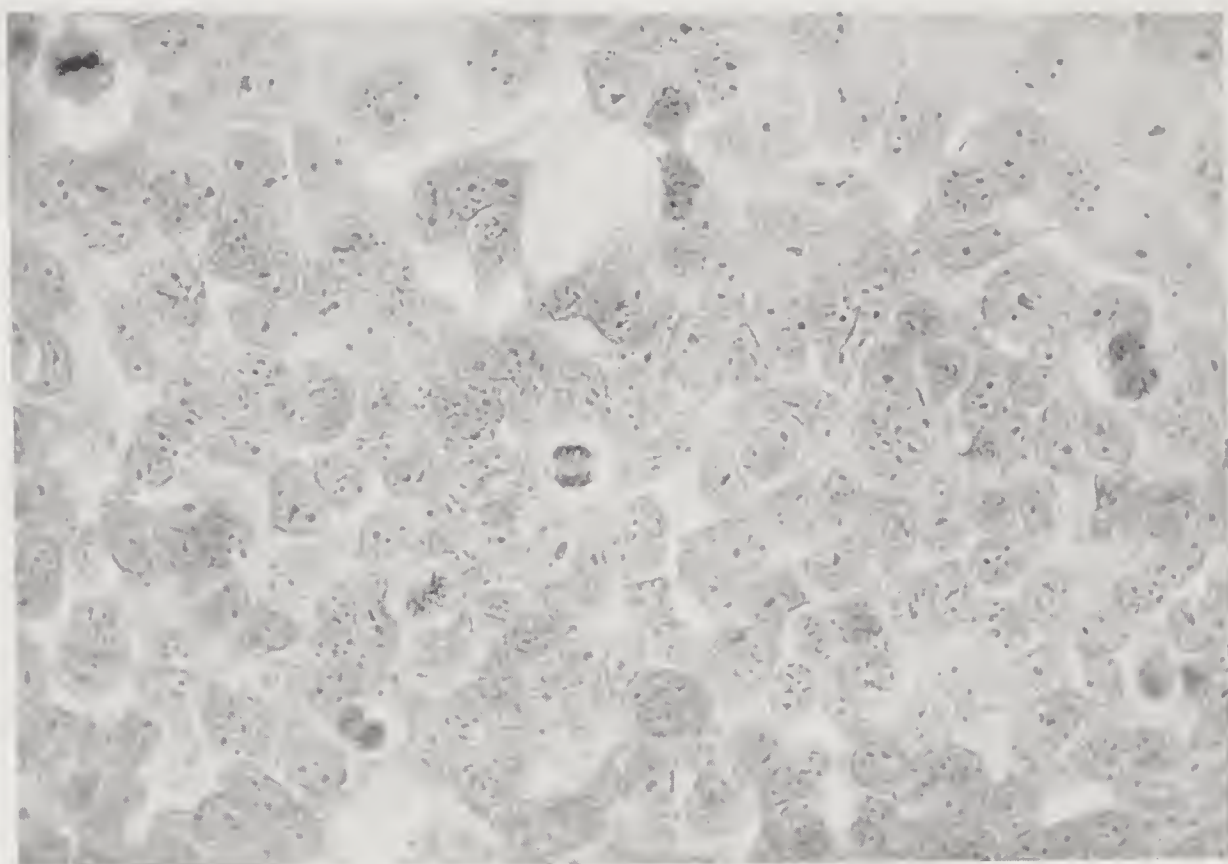


FIG. 1. Mouse carcinoma²⁰⁶ of the Imperial Cancer Res. Fd. (After *Ludford*¹⁴⁹)

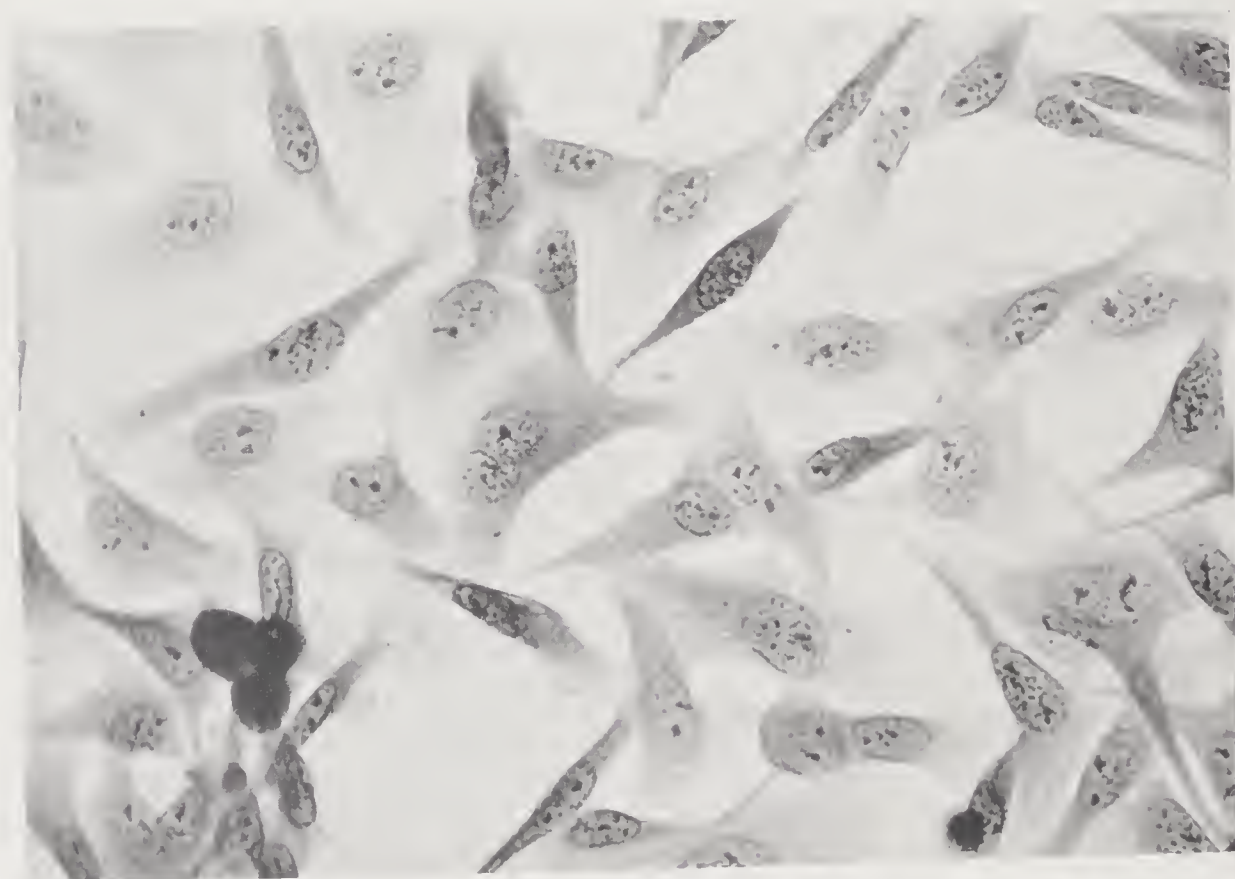
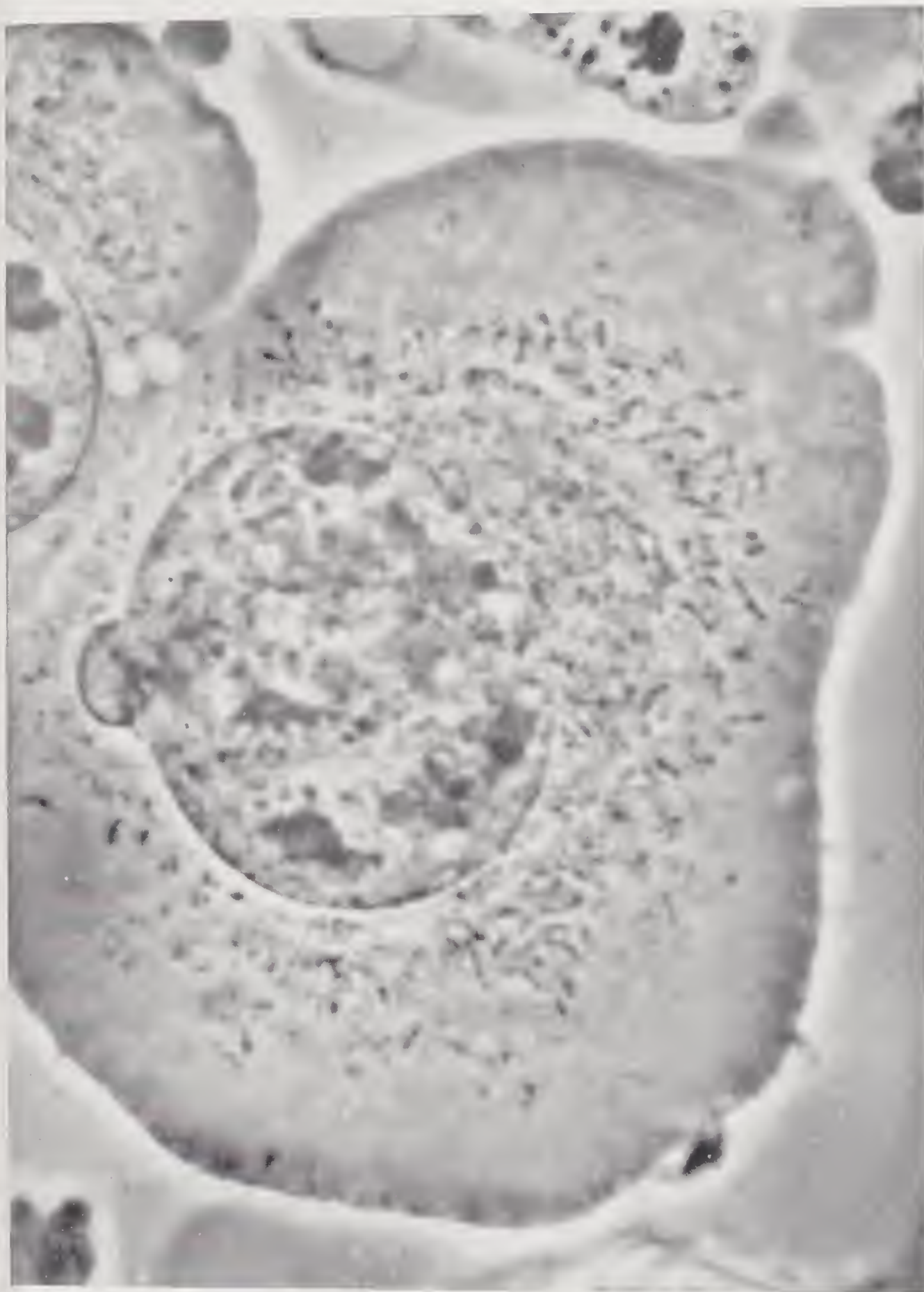


FIG. 2. Jensen rat sarcoma. (After *Ludford*¹⁵⁰)

Characteristic types of growth *in vitro* of a carcinoma and a sarcoma



Phase-contrast photomicrograph of a living malignant cell from a rapidly growing mouse sarcoma. (After *Ludford, Smiles, and Welch*¹⁶²)

R. J. Ludford

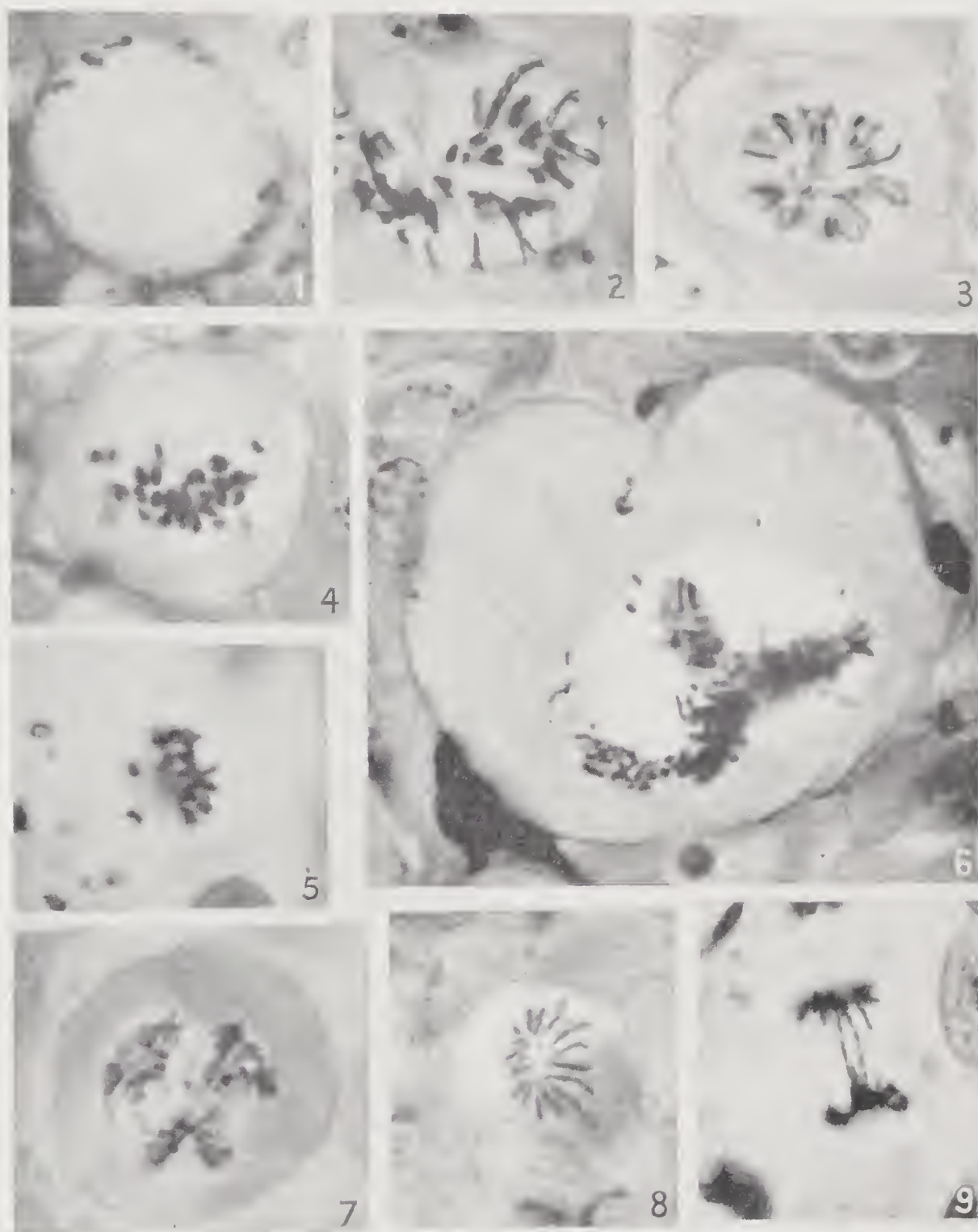


FIG. 1. Chromosome formation without spindle development in Jensen rat sarcoma cell; chromosomes displaced to cell periphery

FIG. 2. Similar aberration in mouse sarcoma 37, but photomicrograph shows surface of cell instead of section as in Fig. 1

FIG. 3. Metaphase in cell of Fibiger's tar tumour; same magnification as cell of the same tumour shown in Fig. 6

FIG. 4. Chromosomes reduced to granular form in mouse carcinoma 63

FIG. 5. Incomplete metaphase plate with two aberrant chromosomes in mouse carcinoma 63. (Original)

FIG. 6. Mitosis in giant cell of Fibiger's tar tumour. More than tetraploid number of chromosomes, some fragmented; deformed multipolar spindle

FIG. 7. Multipolar mitosis in Jensen rat sarcoma growing *in vitro*

FIG. 8. Haploid number of chromosomes in mouse carcinoma 206

FIG. 9. Delayed separation of chromosomes at telophase in mouse carcinoma 63. (Original)

Figs. 1 and 2 after *Ludford*³², Figs. 3, 4, 6, and 8 after *Ludford*³³, and Fig. 7 after *Ludford*³⁴

Aberrations of mitosis in malignant cells



FIG. 1. Well-spread rat fibroblast with large filamentous mitochondria (Original)



FIG. 2. Jensen rat sarcoma cell

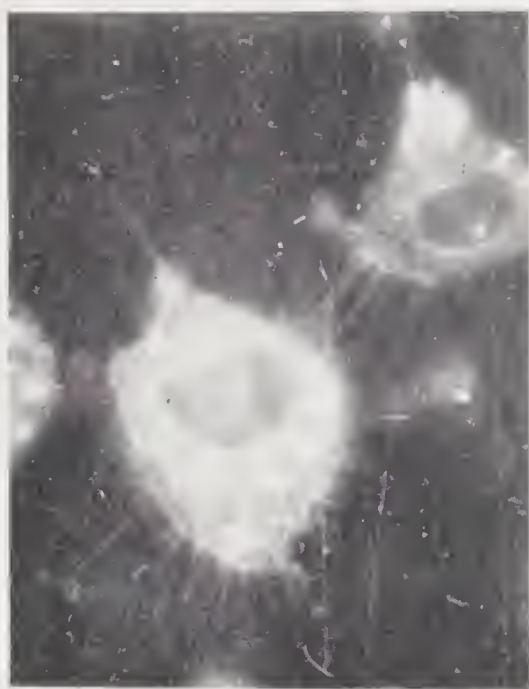


FIG. 3. Crocker mouse sarcoma cell



FIG. 4. Jensen rat sarcoma cell more spread out than in Fig 2. (After Ludford¹⁵⁰)

Photomicrographs by dark-ground illumination of a normal fibroblast and of malignant fibroblasts (sarcoma cells) showing the more granular character of the latter, and their finer mitochondria. All cells photographed in living cultures

R. J. Ludford

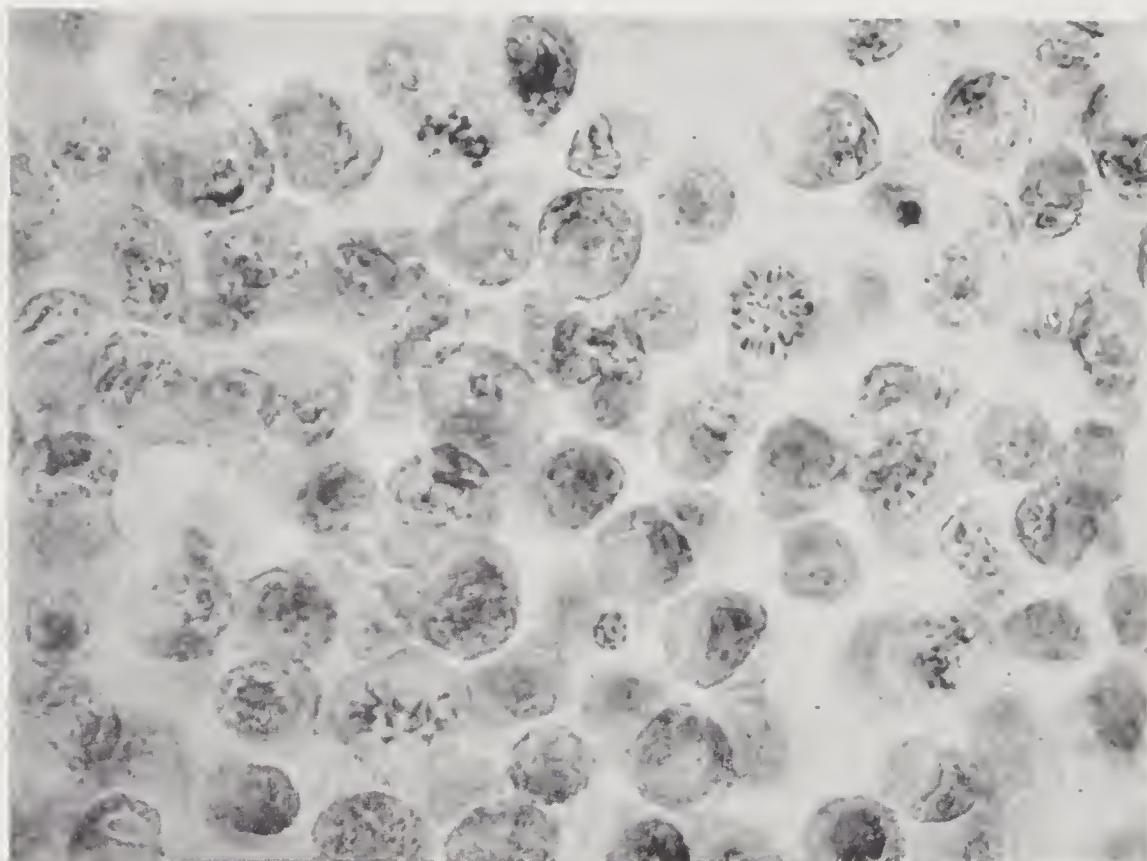


FIG. 1. Rounded cells in serum. $\times 670$. (Ludford¹⁵⁰)

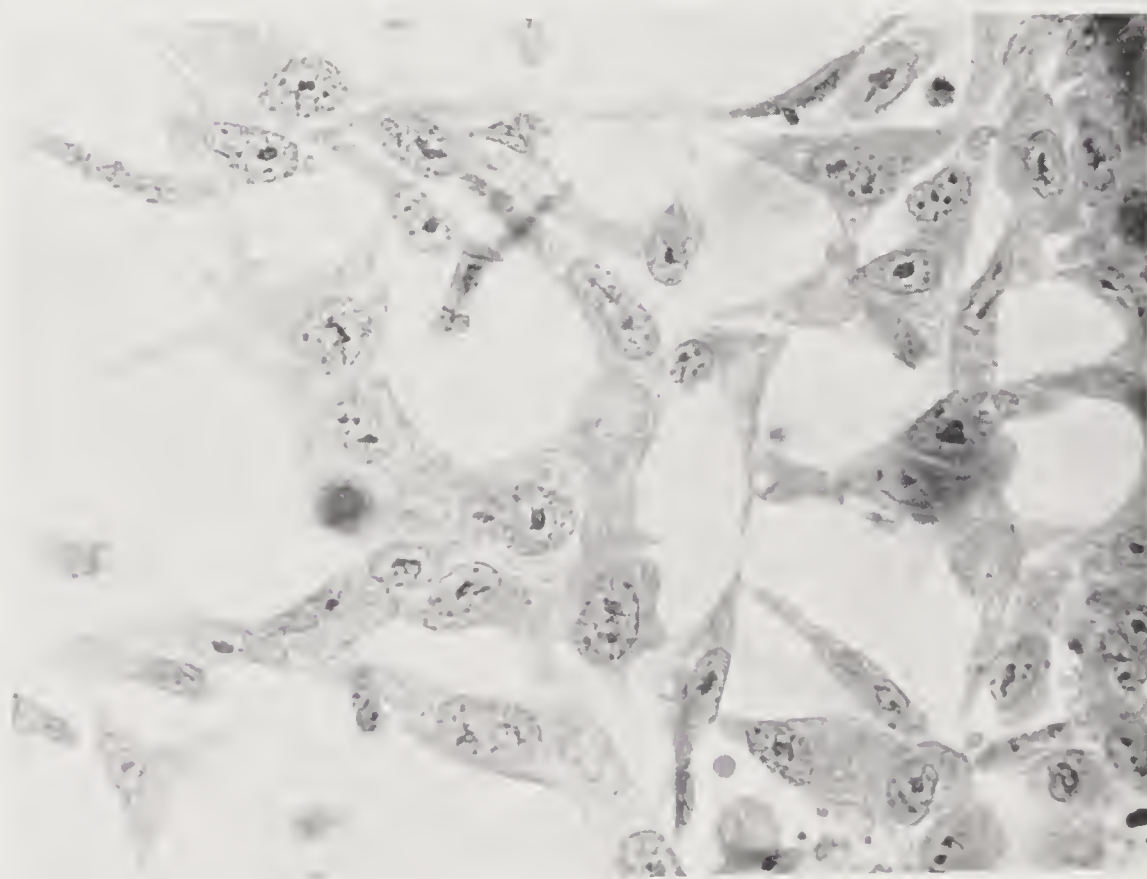
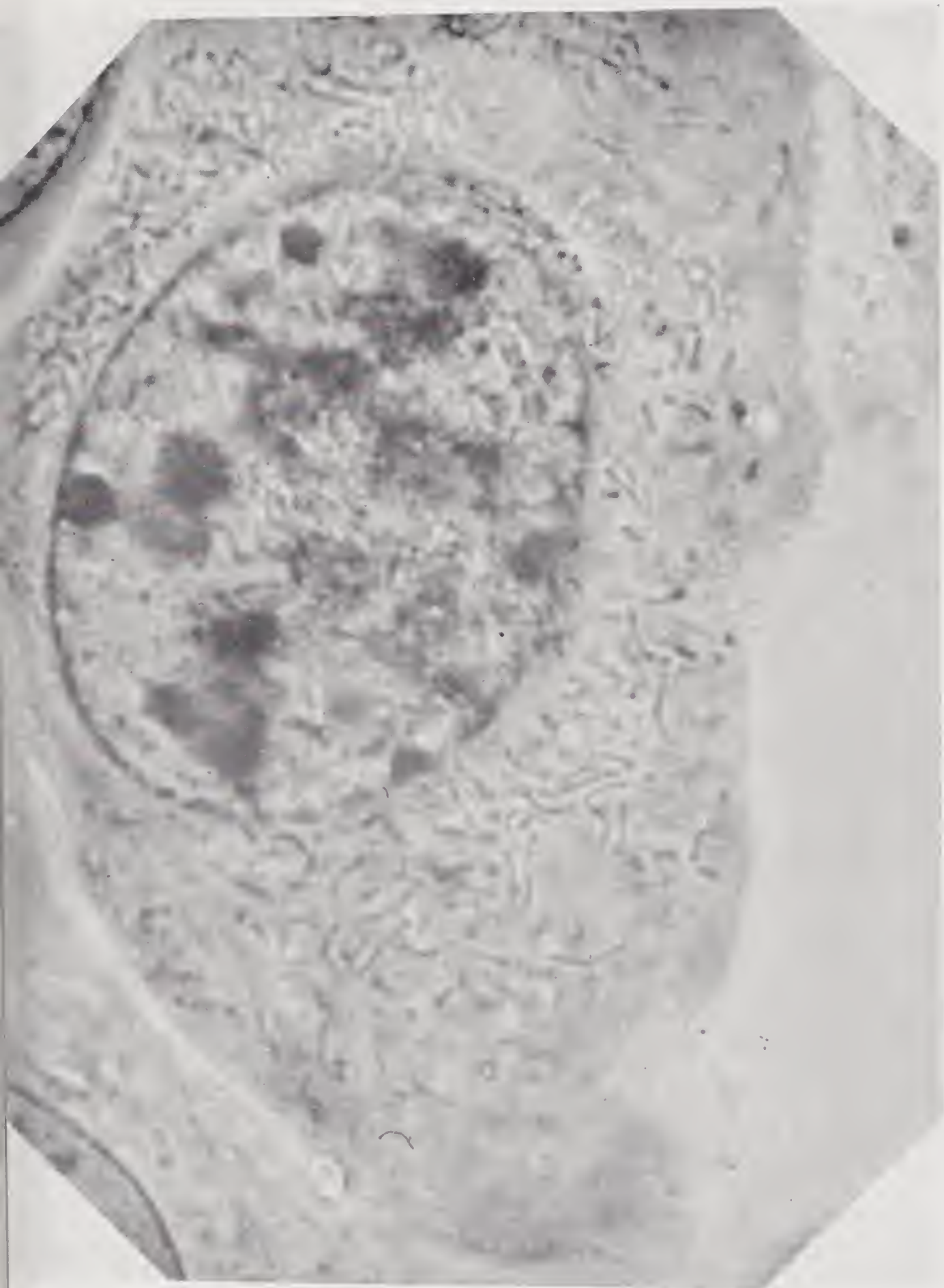
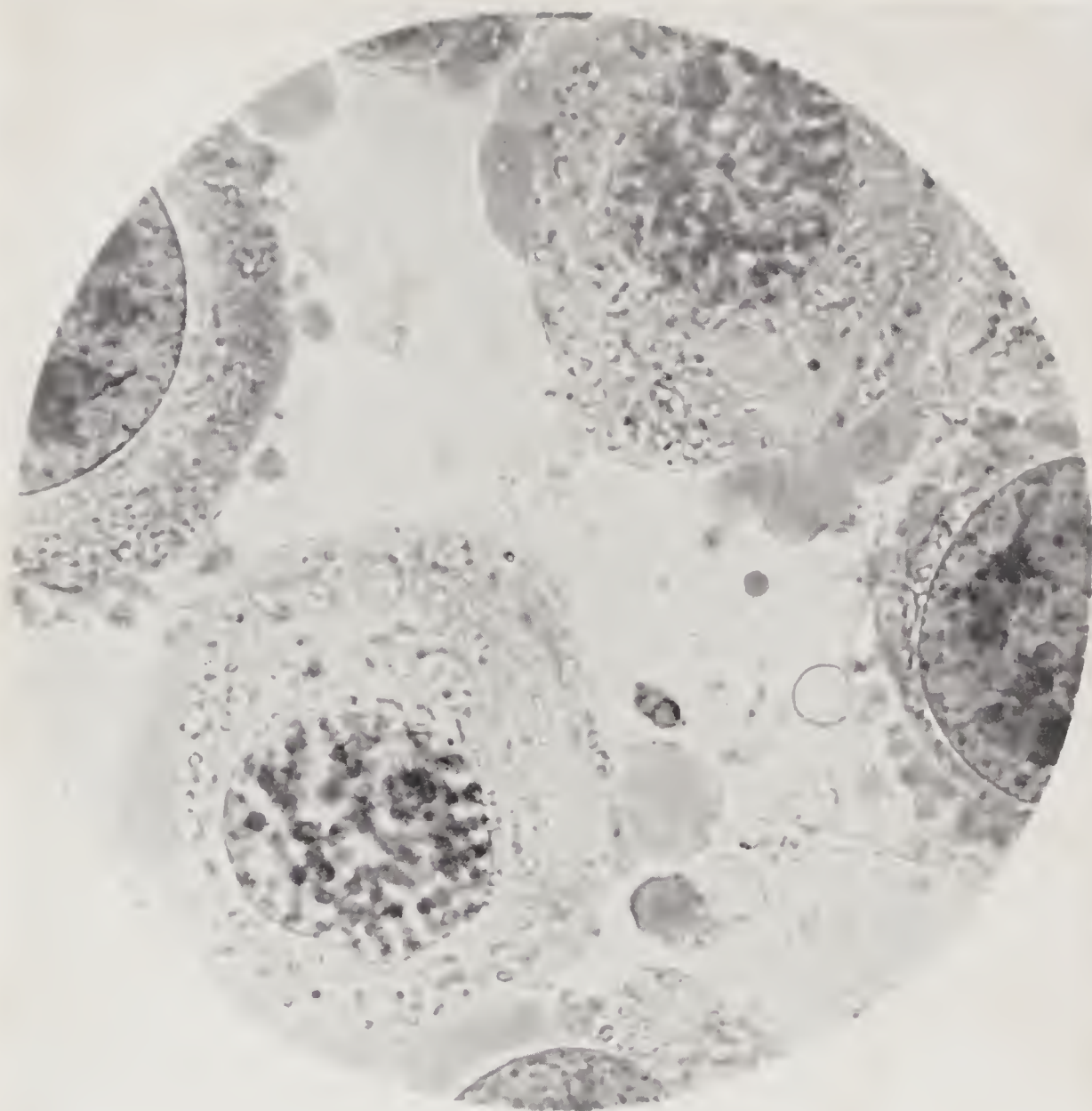


FIG. 2. Well-spread cells growing in plasma clot. $\times 450$. (Original)
Different behaviour of the cells of a sarcoma (mouse sarcoma No. 37) ex-
planted in serum and in clotted plasma



Ultra-violet micrograph of a living sarcoma cell from the same tumour as the cell depicted in Plate 3. 2,750 Å. (After *Ludford, Smiles, and Welch*¹⁶¹)

R. J. Ludford



Termination of mitotic division of a sarcoma cell. Ultra-violet micrograph from a living preparation of the same tumour as the cells of Plates 3 and 7. 2,750 Å
(Original)

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CHAPTER X

HISTOGENESIS IN TISSUE CULTURE

By HONOR B. FELL

i. INTRODUCTION

AS early as 1914, Thomson^{145,146} made the important observation that isolated parts of the chick embryo, e.g. toe, feathers, and lens, enlarged as a whole when cultivated *in vitro*. From this he concluded that 'complete, uninjured portions of chick embryonic tissue grow to some extent *in vitro* under somatic control' (p. 37). Accordingly he applied the terms 'controlled' to this type of growth and 'uncontrolled' to that diffuse out-wandering of cells from a cut surface which is a more familiar feature of tissue culture. Maximow⁷⁸ called the two forms of growth 'organotypic' and 'histiotypic' respectively, and described progressive histological differentiation in the former. Nowadays the two types of growth are usually referred to as 'organized' and 'unorganized'.

In unorganized growth cells migrate actively from the original fragment or explant and multiply in the nutritive medium until the explant becomes surrounded by a broad halo of new tissue. As Thomson¹⁴⁵ pointed out in 1914 (p. 28): 'It is highly probable that the branching growth represents exactly what occurs in a live animal which has received a wound. Plasma is first poured out; this solidifies, and the cells, which are free and exposed, branch into the plasma clot and heal the wound.' By subdivision of the tissue and its repeated transfer to fresh medium, such cultures can sometimes be maintained for years in a state of active proliferation. To a great extent the migrating cells lose their characteristic appearance and histological structure, so that it is sometimes impossible to tell by inspection from which tissue in the body they originated. Some degree of specificity is usually retained, however, and epithelium forms sheets and cords, fibroblasts produce a branching outgrowth, and so on.

Organized growth more nearly resembles normal growth *in vivo*. The tissue largely retains its characteristic architecture, remains functional and, if derived from undifferentiated material, it may develop in culture in a surprisingly normal way. Sometimes this progressive development is purely histological, but sometimes a remarkable degree of anatomical development also takes place.

The work on organogenesis *in vitro* has been reviewed elsewhere²¹, and the present chapter is concerned only with histological differentiation in culture. A brief account will be given of the methods commonly

employed for the cultivation of organized tissues, of the various types of histogenesis that have been observed *in vitro*, and of some of the biological problems for the solution of which cultures of organized tissue have been successfully used.

ii. METHODS

Unorganized and organized growth often take place simultaneously in the same culture. When this happens, organized growth proceeds in the undisturbed interior of the tissue and unorganized growth at the margin. Most tissues differentiate best when cell migration is kept at a minimum because active amoeboid outgrowth naturally tends to disrupt the organization of the tissue, but at the same time fresh food and oxygen must be plentifully supplied.

These conditions can be achieved in various ways. Many tissues differentiate well in ordinary large hanging-drop preparations. The writer uses $1\frac{1}{4}$ -inch square cover-slips mounted over $3 \times 1\frac{1}{2}$ -inch hollow-ground slides, and transfers the tissue to fresh medium, without subdivision, every 2–3 days. In this way the interior of the culture is kept undisturbed, a too-profuse outgrowth is discouraged, and a good nutrition maintained. Another technique which has given excellent results is the double cover-slip method of Maximow^{76,77}. The tissue is explanted into a drop of suitable medium spread on a small round cover-slip, which is then stuck to a large cover-slip by a small drop of saline; the large cover-slip is sealed over a deep hollow-ground slide. Every few days the small cover-slip is detached, washed in saline, and the tissue is re-fed with a small quantity of fresh medium without being disturbed. In this way the explant can be maintained for long periods on the same cover-slip.

Gey's roller tube method^{35,36}, though more generally used for unorganized growth, has been successfully employed for certain types of differentiated tissue^{48,50}. By this technique the tissue is embedded in a thin layer of clotted plasma on the inner surface of a glass tube. Some nutritive fluid, often embryo extract, is added, and the tube is stoppered and placed in a slowly revolving drum. In this way the cells are intermittently bathed with the nutritive fluid, which is withdrawn and replaced at suitable intervals, the tissue being left untouched for many days.

For cultivating relatively large rudiments the watch-glass method is useful²⁴. This is based on a technique originally devised by T. S. P. Strangeways (unpublished). A watch-glass is enclosed in a Petri dish carpeted with wet cotton-wool in which a circular hole has been cut to permit transillumination of the explant. The medium, in the form of a semi-solid clot, is contained in the watch-glass, and the tissue is explanted on the surface. Every 2–3 days the explants are loosened from the clot, washed, and transferred to freshly prepared medium in a clean

watch-glass. The advantages of this method are that the explants are so readily accessible to surgical manipulation²² and that, as stated above, relatively large masses of tissue can be grown. It is unsuitable for very small explants, and has the further disadvantage that the tissue cannot be studied under high magnifications.

Most tissues, both avian and mammalian, differentiate well in a mixture of fowl blood-plasma and chick embryo extract. The relative proportions of plasma and embryo extract which should be used depend on the tissue to be cultivated and the culture method employed, and must be determined by trial and error for every new tissue. It is important that the embryo from which the extract is made should not be too young, a point which has been clearly demonstrated by Gaillard and his pupils³², to whose work further reference will be made later. Other ingredients may be added to the culture medium for different tissues, e.g. mammalian plasma or serum, human cord serum, &c.

Another factor which is often important in obtaining successful differentiation *in vitro* is the age of the embryo from which the tissue is taken. For many tissues there is an optimum stage of development at which the explants should be taken, and either younger or older material gives unfavourable results for reasons which are not yet understood.

iii. HISTOGENESIS

The different types of histogenesis that have been observed *in vitro* will now be described.

a. Connective Tissue Fibres

The differentiation of fibrous connective tissue (Plate 1, Fig. 1) has been studied quite extensively in both avian and mammalian material^{1,3,4,12,46,63,64,69,79,80,81,87,108,110,112,113,120,121}. Collagen is formed *in vitro* by fibroblasts from many different sources, e.g. spleen, lymph nodes, embryonic subcutaneous tissue, heart, liver, &c. As the fibroblasts migrate from the explant, a fine fibrillar network of argyrophil reticulum develops around the cells, sometimes during the first 24 hours' growth⁸⁷. This reticulum, which is visible in the living culture, becomes rapidly transformed first into a coarser network and then into large fibres; the transformation is effected partly by the mechanical orientation of the fine fibrils by the migrating cells, and partly by their conglutination into thicker elements. Sometimes the fibres in the zone of outgrowth form in direct continuity with those of the original explant^{3,81}, and sometimes they develop independently. Since the new fibres are more profuse near the original explant, some authors consider that their production is stimulated by the presence of the pre-existing collagen^{3,46,120}.

Whether collagen fibres originate inside or outside the cell has long

been a subject of controversy among histologists. Doljansky and Roulet¹² performed the interesting experiment of growing fibroblasts on a porous glass plate above a plasma clot, so that the cells were not in direct contact with the medium although their products could diffuse into it. Bundles of fibres formed beneath the porous plate, presumably by an interaction between the culture medium and substances secreted by the overlying fibroblasts. These fibres stained deep blue with azan, but gave no reaction with Weigert's fibrin stain; they became increasingly resistant to trypsin digestion as their development progressed, and although this resistance was never complete, it was as great as that of collagen formed in tissue cultures in the ordinary way. These results suggest that although collagen fibres are formed as a result of cellular activity, direct contact with the cells is not essential.

The relationship of fibrin to the formation of collagen has also been a matter for dispute. Baitsell¹, working with cultures of adult frog tissue, believed that fibrin was directly transformed into collagen, but it has since been shown⁸⁷ that collagenous fibres are able to develop in a fluid medium devoid of fibrin.

The formation of elastic fibres in culture has been noted by several workers^{4,97,98,112}. Such fibres only develop *in vitro* in those tissues which normally form them *in vivo*. They have been very clearly demonstrated in cultures of the embryonic heart muscle of the guinea-pig⁴. The elastic fibres, which gave the specific staining reaction with Weigert's resorcin fuchsin, reached their highest development in the zone of outgrowth, and were thickest and most prominent where they were subjected to the pull of the pulsating cardiac muscle. Elastic fibres also form in non-pulsating cultures, however, showing that the contractile force is not essential for their development^{4,97,98,112}.

b. Skeletal Tissue

The differentiation of hyaline cartilage can be watched day by day in living cultures of chondrogenic mesoderm^{17,52,96,119,129}. If a small piece of the undifferentiated axial tissue is removed from the limb-bud of a 3-day chick embryo¹⁷ and cultivated in a large hanging-drop culture, it chondrifies within a few days. During the first 48 hours the explant rapidly expands into a sheet in which slight local thickenings are seen. These condensed areas enlarge, become more compact, and by the 4th day delicate partitions of glistening matrix have appeared between the polyhedral cells, which are now recognizable as young chondroblasts. The intercellular material steadily increases in amount, and the young cartilage becomes sharply demarcated from the surrounding mesoderm by a fibrous perichondrium. If the axial mesoderm of limb-buds is grown by the watch-glass method, it undergoes a surprising degree of anatomical development, including the early stages of joint-formation²².

Under appropriate conditions, cartilage which ossifies in the embryo also ossifies in culture^{17,24,25,29,32,83,85,86,118}. For example, the early cartilaginous rudiment of the chick femur, when grown by the watch-glass method, differentiated to a surprising degree²⁴. When first explanted it consisted of small-celled cartilage with no demarcation into epiphysis and diaphysis and no periosteum. During cultivation the cells of the shaft began to hypertrophy in the normal way, the perichondrium differentiated into an inner osteoblastic and an outer fibrous coat, and a sheath of osteoid tissue was deposited on the surface of the hypertrophic cartilage; cellular hypertrophy spread from the centre of the shaft towards the ends, but it stopped abruptly at the junction of the epiphysial and diaphysial regions so that, as in the normal avian embryo, the small-celled epiphysis was sharply demarcated from the large-celled shaft. Meanwhile the layer of osteoid tissue thickened and calcified to form true periosteal bone. The gross anatomy of the rudiment also developed to a remarkable extent.

Robison and his pupils have shown that in the normal animal hypertrophic cartilage and bone both secrete large quantities of alkaline phosphatase. At the stage when the femur rudiments were first explanted they contained little or no phosphatase, but as they differentiated during cultivation the enzyme was secreted as actively in the cultures as in the normal developing femur *in vivo*²⁴. There was thus a biochemical as well as a morphological differentiation *in vitro*.

If such long-bone rudiments are fractured, the fractures are repaired *in vitro*⁹⁵. The type of repair depends on the period of embryonic life at which the injury is made. If the rudiments are taken from 5-day chick embryos, when they consist of very early unossified cartilage, the cut surfaces of the fracture rapidly unite by proliferation of the chondroblasts, continuity of the perichondrium is restored, and within a few days no trace of injury remains. At a slightly later stage there is no direct fusion of the cartilage, but the young periosteum quickly heals and osteoblasts often migrate between the cut surfaces of the cartilage, where they sometimes form osteoid tissue; a continuous sheath of bone is deposited, completely covering the site of fracture. When ossified rudiments are fractured the periosteum is soon repaired, osteoblasts fill the gap between the cut ends, and the periosteal bone is healed by fresh deposition.

At certain stages of development the differentiation of cartilage which normally ossifies can be arrested by appropriate environmental conditions, so that chondroblastic hypertrophy and ossification are completely or partially suppressed, although the cartilage appears healthy²³. This was shown by the following experiments. The skeletal rudiments were removed from the leg-buds of normal chick embryos at different stages of development; one of each pair was grown in normal culture

medium and the other in a diluted, growth-restricting medium. Cultivation *in vitro* always greatly inhibits skeletal growth, but this inhibition was much more exaggerated in the diluted medium. In the youngest group of these paired rudiments (leg-buds 0.48–2.19 mm. in length) 13 per cent. ossified in the normal and only 3 per cent. in the growth-restricting medium; in the next group (2.24–3.81 mm. buds) 73 per cent. ossified in the normal and 27 per cent. in the growth-restricting medium, while in the oldest group (3.86–5.24 mm. buds) all the explants ossified in both media. After 16–18 days' cultivation in the diluted medium the explants from the younger embryos resembled the long-bones of phokomelic chick embryos in the following respects: retardation of chondrogenesis, absence of ossification, fusion of tibia and fibula, and the absence of chondroblastic hypertrophy or its restriction to the interior of the shaft. These results indicated that there is a critical period in the early development of the normal limb-skeleton during which conditions that restrict growth also prevent subsequent ossification, but that after this period the same conditions fail to inhibit ossification.

Another important factor in the development and ossification of cartilage is the age of the embryo from which the tissue extract for the culture medium is made. It was shown (Verdam, quoted from ³²)^{83,85} that when cartilaginous rudiments were explanted at a rather later stage than the critical period mentioned above, the degree of ossification which the cultures underwent depended on the age of the embryo from which the tissue extract was prepared. Thus when tibiae from 7-day chick embryos were cultivated in a medium containing extract of 7-day embryos, the cultures grew well but did not ossify; on the other hand, they ossified readily when an extract of 13-day embryos was used^{83,85}. Extract of 18-day embryos was somewhat toxic unless it was twice centrifuged, in which case it promoted both cartilage growth and the formation of bone. The metacarpals of rat foetuses cultivated in medium containing press juices of younger embryos quickly and completely lost their histological structure, and the cartilage dedifferentiated into a shapeless mass of cells resembling those of connective tissue (Verdam, quoted from ³²); the histological structure of metacarpals grown in extract of embryos of the same age as those from which the explants were taken remained almost unchanged, but in blood-plasma from adult rats both differentiation and ossification progressed. The best results were obtained when the rudiments were cultivated by Gaillard's 'ascending range' method, i.e. when at each subculture the metacarpals were transferred to medium containing the press juices of an embryo older than that used for the preceding passage³².

In the normal embryo there are two main types of cartilage: (1) hypertrophic cartilage with large chondroblasts, which forms alkaline phosphatase and is always associated with periosteal bone, and (2) non-

ossifying small-celled cartilage which contains little or no phosphatase²⁵ and does not ossify, e.g. Meckel's rod and the avian epiphyses. We have seen that cartilage which normally undergoes cellular hypertrophy and ossification *in vivo*, under favourable conditions differentiates in the same way *in vitro*. The small-celled cartilage also develops *in vitro* as it does *in vivo*. For example, if the palato-quadrate and Meckel's rod are removed from a 5-6-day chick embryo, at which stage both are small-celled and unossified, and are explanted *in vitro*²⁵, the former undergoes chondroblastic hypertrophy, synthesizes phosphatase and ossifies, while the latter remains small-celled and forms no phosphatase throughout the period of cultivation. From this it is clear that the future histological structure of the two types of cartilage is determined before any significant histological difference between them has appeared.

The question has often been raised as to whether bone is formed by ordinary connective tissue cells in response to local humoral conditions or by specifically determined, self-differentiating osteogenic cells. This problem was investigated by studying the osteogenic capacity of isolated *periosteum* and *endosteum* when growing under the standardized environmental conditions obtaining in tissue culture¹⁹.

In these experiments the periosteal tissue was stripped from the shafts of long-bones from 6-21-day fowl embryos and from newly hatched chicks and cultivated in large hanging-drop preparations. The explants from 6-day embryos ossified readily *in vitro*, and the details of bone formation could be followed day by day in the living tissue. After 48 hours *in vitro* the explants had expanded into very delicate sheets of tissue bordered by a fringe of amoeboid cells and containing one or more compact thickenings. The cells of these denser regions were somewhat rounded, and were separated from each other by delicate, slightly refractile and apparently homogeneous partitions. After 4 days some of these thickenings had developed into nodules of typical hyaline cartilage, while others, sometimes in the same culture, had given rise to ossification centres. In the latter, fine osteogenic fibres appeared among the cells, forming rather irregular, very refractile branching bundles. When the tissue was transplanted to fresh medium it was found that this early intercellular material had a certain rigidity even in the thinnest ossification centres, so that when the tissue was cut out of the plasma clot it remained as a fairly flat plate around which the soft mesoderm contracted into a crumpled ring. In the 6-day cultures the fibrous structure of the osteoid matrix was obscured in places by the deposition of a refractile, interfibrillar cement, presumably ossein. This material rapidly calcified so that the bone became white and opaque in the interior of the ossification centre, though new osteogenic fibres radiating into the tissue in all directions were often in course of

formation at the periphery. Many ossification centres appeared healthy after 18 days' cultivation. Histological sections of such cultures showed typical coarse-fibred bone (Plate 1, Figs. 3 *a* and *b*) indistinguishable from that formed in the normal embryo.

Periosteum from 10-day embryos also ossified well in culture, forming rather thicker and more compact ossification centres than those described above. In the early experiments ossification was rarely obtained from the periosteum of late embryos and newly hatched chicks, although the tissue grew profusely. It was found that this was due merely to mechanical damage inflicted on the delicate osteoblastic layer when the tough, closely attached fibrous coat was stripped from the bone. The outgrowth in these early cultures was therefore fibroblastic and non-osteogenic. In later (unpublished) experiments, in which the technique of dissection had been improved, the older periosteum ossified *in vitro* almost as readily as the younger tissue.

To study the bone-forming capacity of the endosteum, fragments of tibial bone from a late embryo or newly hatched chick were cleaned of periosteum and marrow, and grown by the hanging-drop method for 5-6 days; by this time the bone had become surrounded by a broad halo of outgrowth, consisting mainly of endosteal osteoblasts which had crawled out of the Haversian spaces and multiplied in the medium. The original fragment was then excised and rejected and the zone of outgrowth subcultivated. New bone soon developed in the interior of such endosteal cultures (Plate 1, Figs. 2 *a* and *b*). Sometimes cartilage also differentiated¹⁸; when it appeared it was always of the hypertrophic type, produced but little chondroid matrix (which, however, stained metachromatically with thionin) and merged gradually with the surrounding bone and osteoid tissue. The general histological picture closely resembled that of the chondroid areas in callus. This low-grade cartilage was unstable and usually became transformed into osteoid tissue and bone.

Membrane bone differentiates well *in vitro*²⁵. The undifferentiated mesoderm surrounding Meckel's cartilage in the 6-day chick embryo was cultivated by the hanging-drop method and formed nodules of typical calcified bone, the development of which was beautifully clear in the living cultures.

The results of these tissue-culture experiments strongly support the view that, at least during embryonic and early post-embryonic life, bone is formed by specifically determined cells and not in response to local environmental factors. That fibroblasts can be transformed into osteoblasts under certain abnormal conditions is not questioned, however, since it is well known that ectopic bone can be produced experimentally or under pathological conditions in various tissues and organs *in vivo*.

This specificity of the skeletogenous mesoderm and its remarkable capacity for self-differentiation *in vitro* have been utilized for analysing the developmental potencies of undifferentiated regions of the early embryo. For example, the processes which lead to the formation of the mesodermal condensations which give rise to the cartilage and bone of the chick mandible were investigated by explantation experiments⁵². The potencies of different parts of the early mandibular rudiment at different stages of development were ascertained by excising selected regions of mesoderm and allowing them to differentiate *in vitro*; each mandible from which an explant had been taken was fixed and sectioned, so that the precise boundaries of the excised fragments could be determined. The results showed that the chondrogenic cells which form Meckel's cartilage originate from a small proliferation centre immediately beneath the buccal epithelium of the branchial arch; the cells of this area are already determined to form cartilage by the third day of embryonic life (Plate 1, Figs. 4 *a* and *b*). The osteogenic cells destined to form the angulare, spleniale, and supra-angulare were found to arise from a single proliferation centre, which appears at about the fourth day beneath a slight ectodermal thickening at the proximo-lateral end of the mandible; the explantation experiments demonstrated that even at this early stage the cells of the centre are determined to form bone. The os dentale originates from a separate centre, which appears at about the fifth day in the disto-lateral part of the mandible.

Similar experiments were made²⁰ to find whether the avian sternum was derived from the ribs, as some anatomists have maintained, or whether it should be regarded as part of the appendicular skeleton, as others believe. It was found that the sternal plates can develop *in vitro* in the complete absence of both ribs and coracoid. The boundaries of the presumptive sternal tissue were determined by excising different regions of the lateral body wall and studying their development *in vitro*; it was clear that the plates arise as independent structures which, however, are more closely related to the appendicular than to the axial skeleton. In rather older explants of the ventro-lateral body wall it was demonstrated that, as in the intact embryo, there is a general movement towards the mid-line of all the dorso-lateral tissue, including muscle, connective tissue, and the sternal plates.

Under certain conditions, tissue of skeletal origin which has completely lost its original structure during weeks or months of repeated subculture can be made to re-differentiate. Fischer and Parker²⁷ maintained a strain of cultures derived from the cranium of a 10-day chick embryo for several months, by which time all differentiation had been lost. When such cultures were grown for a few passages without embryo extract, a fibrous differentiation occurred in the interior of the tissue which, in the authors' opinion, sometimes resembled bone. Gaillard³²

obtained rather more definite results when he induced completely de-differentiated osteoblast cultures to form typical calcified bone by means of his 'ascending range' method mentioned above, i.e. by sub-cultivating the tissue in medium containing press juices from successively older embryos.

The effects of mechanical factors on skeletal structure have been studied in cultures of cartilage and bone^{39,40,41,150,151}. As Glücksmann⁴¹ points out, in animal experiments it is almost impossible to define the minute stresses set up in skeletal tissue, as the physical conditions are so greatly complicated by the presence of muscles, nerves, and blood-supply. The direct effect of various known stresses on the histological architecture of skeletal tissue can therefore be studied with much greater precision *in vitro*, where these complicating factors are eliminated.

In one series of experiments⁴⁰ mechanical forces were applied to the skeletal explants in three ways: (1) The direct application of pressure. Small sections of two adjacent ribs from 17–21-day fowl embryos, connected by the intercostal muscle, were explanted by the hanging-drop method, and the experimental culture was laid on the muscle between the ribs; during cultivation the rib-fragments were drawn together, thus exerting pressure on the explant lying between them. (2) The indirect application of pressure. Cartilaginous barriers were placed in contact with the growing culture so as to resist its expansion at selected points. (3) By the surface tension of the culture medium. If tissue is explanted near the margin of a hanging drop the centre of the clot liquefies, while the periphery loses fluid and shrinks, thus increasing the surface tension near the margin.

When endosteal cultures such as those described on p. 426 were subjected to direct pressure³⁹, they acquired an orientated structure; whether this was of the resistance or compression type depended on the degree of calcification of the tissue at the time when the pressure was applied. Long-bone rudiments also were subjected to mechanical forces with the following results. If the perichondrium or periosteum were exposed to pressure or tension stresses acting at right angles to the direction of the fibres, the collagen fibres disappeared and cartilage was formed⁴⁰. When pressure or tension was applied to differentiated cartilage⁴¹ the hyaline matrix disintegrated and was replaced by a fibrillar system. Periosteal ossification was promoted by tension stresses, which also determined the pattern of the osseous architecture.

Other experiments were made on the effects of mechanical stresses on the differentiation of the scleral cartilage of chick embryos^{150,151}. When explants of early scleral cartilage were folded, the distribution of the cells and the orientation of their nuclear axes reflected very clearly the pattern of the lines of stress. The perichondrium was dense on the convex surfaces, which were under tangential strain, and the

cells and fibres ran parallel to the surface of the cartilage; on the concave side it became loosened into a stroma, the fibres of which radiated into the interior of the scleral cartilage along the lines of tension produced by the lateral compression of the plate.

The effect of tension on the differentiation of scleral cartilage was studied in the following way¹⁵⁰. The partly coagulated plasma clot containing an explant of pre-scleral mesenchyme was stretched with a needle and held until coagulation was complete; the clot then adhered firmly to the cover-slip, retaining its deformation and resulting elastic tensions. If the stretch applied to the scleral mesenchyme was excessive, the explant was drawn out into a thin layer in which heavy bundles of collagen developed along the lines of stress, but no cartilage. With moderate stretch, cartilage developed but was abnormally thin.

From all these investigations it is clear that the effects of mechanical stresses on skeletal tissue developing under the greatly simplified conditions of culture *in vitro* closely resemble those produced by similar mechanical factors *in vivo*. Evidence is thus provided that the structural effects produced *in vivo* are due to the direct action of the stresses on the skeletal tissue itself.

c. Teeth

Tooth germs have as great a capacity for differentiation *in vitro* as skeletal rudiments^{37,38}. Molar tooth germs of the rat and rabbit were explanted before cusps had appeared or dentine and enamel had differentiated. During cultivation cusps developed almost normally, the dentine papilla formed odontoblasts which deposited normal tubular dentine, and in the rabbit tooth rudiments the internal enamel epithelium differentiated into ameloblasts which formed enamel. It was found that the presence of the internal enamel epithelium was essential for the differentiation of the odontoblasts, but that when once the odontoblasts had appeared they could produce dentine in the absence of the enamel epithelium. The results also showed that the enamel epithelium was responsible for the normal anatomical development of the tooth.

d. Skeletal Muscle

The development and behaviour *in vitro* of skeletal muscle has been studied extensively. Cross-striated muscle fibres will develop in culture from mononucleate myoblasts, whether the explants have nervous connexions or not^{52,136}. Cultures of embryonic muscle proved convenient material for investigating the physiology of contraction at different stages of myogenesis^{122,123,133-141}.

In one set of experiments¹³³ explants consisting of 1-4 myotomes with the corresponding piece of spinal cord were removed from 24-42-

hour chick embryos and cultivated *in vitro*; both muscle fibres and nerves differentiated. At first only a few isolated myoblasts contracted, but the activity spread rapidly and soon all the muscular tissue was involved. The early sporadic contraction seemed to be myogenic, but the later contractions appeared to be neurogenic and could be suppressed by curare. Histological preparations showed that by the time muscular contraction was active the reflex arc was established throughout the preparation¹³⁵; in those explants where there was no muscular activity the reflex arc had not yet been formed. From these results it was concluded that the contractions were reflex actions. Cultures of myogenic tissue completely isolated from all nerve connexions also develop spontaneous contractions, but require a longer time than the innervated explants to acquire the same degree of activity^{136,141}.

Recently, experiments were made to find whether there is any relationship between electrical excitability and the appearance of spontaneous muscular activity¹⁴¹. Innervated and non-innervated cultures of myogenic tissue were submitted to condenser discharge shocks, and the threshold of response at different stages of differentiation was determined. The threshold was found to decline as differentiation advanced and became low when spontaneous activity appeared; the decline was more rapid in innervated than in non-innervated explants; when the spontaneous contraction of innervated explants was stopped by curare, the threshold simultaneously increased by 10–15 times. From these results it seems likely that the appearance of spontaneous contractions in an explant depends in part upon the degree of its excitability.

In cultures of partly or fully differentiated muscle, some or all of the fibres dedifferentiate, losing their cross-striation and obvious myofibrils^{51,58,61,65,71,115}, but it is interesting that such fibres remain contractile. The general histological picture in these cultures resembles that seen in regenerating muscle *in vivo*. In addition to ordinary spindle-shaped myoblasts, large multinucleate ribbons or irregular masses of protoplasm grow out as buds from the cut ends of the original fibres. These buds, which often become detached from the parent fibre and lead an independent life, may break up into individual cells^{61,115,153}, or they may re-differentiate into cross-striated fibres^{9,61} capable of rhythmical contraction⁹.

The growth *in vitro* of adult rat and human muscle (Plate 2, Fig. 6) has been described¹¹¹. Spindle-shaped cells, irregular multinucleate fragments, and ribbon forms appeared in the zone of outgrowth, and spontaneous contraction was widespread in the rat tissue, though it occurred in only one human culture. Cross-striation was an unstable feature of the muscle cells and in the rat cultures would appear and disappear more than once in the same cell; contraction was seen in both striated and unstriated fibres.

e. Heart Muscle

There is an extensive literature on the behaviour of cardiac muscle *in vitro*^{5,11,42,47,48,57,66,67,70,91,92,93,99-107,116,127,139,140,141,149}, and the contributions up to 1934 have been fully reviewed by Levi⁵⁹. It has been shown^{104,141} that the presumptive heart tissue from 24-30-hour chick embryos rapidly forms myoblasts when cultivated *in vitro* and soon begins to beat, the contractions persisting for about a month. If the explants are grown in medium containing KCl they grow but do not pulsate, but nevertheless cross-striated muscle fibres develop, showing that differentiation does not depend on functional activity.

The threshold of excitability of early embryonic heart muscle explanted *in vitro* has been determined¹⁴¹ by leaving the cultures at room temperature for a short time and, as soon as they lost their spontaneous activity, submitting them to condenser discharge shocks. Developing cardiac muscle was found to differ from developing skeletal muscle in two important respects: (1) the threshold was low from the beginning of the culture period, (2) it did not decline appreciably as differentiation advanced. Since heart muscle is very precocious in its activity, these results provide further support for the view that spontaneous contraction depends largely on the excitability of the muscle.

Pulsating explants of embryonic heart have been used to study the effects of various salts on heart-beat^{91,92,93,99}. The explanted heart rudiment of early chick embryos undergoes a co-ordinated, rhythmical pulsation in normal medium, but if excess potassium, calcium, or magnesium is added, co-ordination is lost and a fibrillation is produced which sometimes lasts for several weeks⁹³. Co-ordinated beating is resumed if the proper balance of the medium is restored. The effect of various drugs and hormones on heart cultures has also been investigated¹⁴⁹.

When heart muscle is explanted at a more advanced stage of development it usually dedifferentiates and loses its cross-striation^{69,103}. Pulsation continues, however, showing that contractility does not depend on the visible fibrillar structure. Under appropriate conditions the myoblasts may re-differentiate and the cross-striated myofibrils reappear⁴², but the character of the contraction is unaffected by these histological changes.

With suitable methods of cultivation cardiac muscle can be kept in a functional and differentiated state for relatively long periods. Embryonic chick heart muscle, for example, when grown in a special fluid medium⁴⁷ remained alive and differentiated for 180 days. Recently⁴⁸ advanced differentiation of chicken cardiac muscle has been obtained by the roller tube method; the fibres became cross-striated, and after about 20 days' cultivation intercalated disks appeared and remained visible until the end of the experiment (60 days).

f. Smooth Muscle

Smooth muscle from various sources has been successfully cultivated in a differentiated and functional state^{43,60,68}. Myofibrils have been beautifully demonstrated in the living embryonic cells by polarized light⁶⁰; the myoblasts contract, either singly or in groups, and sometimes only part of a cell is contractile⁶⁸. Experiments have been made to find when contractility develops in the smooth muscle of the various tissues of the chick embryo, by cultivating explants from the oesophagus, intestine, aerial sacs, and large blood-vessels⁴³. Contractility did not develop simultaneously in the different organs and, as in skeletal and cardiac muscle, it appeared before visible myofibrils had differentiated. Smooth muscle has some capacity for self-differentiation, but according to Gossi⁴³ this is more limited than in embryonic myocardium.

g. Blood

Although erythropoiesis takes place *in vitro*, for some reason it does not remain active in culture for more than a few days. It has been recorded in a variety of embryonic and adult tissues from different species^{2,10,15,26,30,44,53,89,90,114,124,125,152}. Perhaps the most favourable material for observing the details of erythropoiesis in living tissue is provided by small explants of the presumptive erythrocytic mesoderm of an early chick blastoderm⁸⁹. During the first 24 hours the mesenchyme in the interior of such a culture condenses into a mass of actively dividing, polyhedral cells—the haemangioblast, surrounded by a sheet of large, vacuolated epithelial cells. The erythrocytic cells rapidly differentiate into small, almost spherical primitive blood cells and the haemangioblast, or blood island, instead of being flattened on the glass becomes rounded, and in life ‘looked like a pile of round, glistening grapes’ (⁸⁹, p. 504). Soon after this stage the blood island disintegrates, shedding the blood cells into the culture medium, where they continue to develop. The small, primitive blood cells enlarge and assume their characteristic oval form, but these primary erythrocytes seldom develop into mature erythrocytes. Haemoglobin has been demonstrated spectroscopically in the cultures but is often visible to the naked eye.

Different parts of the blastoderm were isolated and their capacity to form blood *in vitro* was ascertained. In this way the region occupied by the tissue pre-determined to form haemangioblasts was defined.

Fragments of the area opaca survive in physiological saline alone, and form epithelial vesicles in which haematopoiesis takes place⁹⁰; if the young blood cells escape into the saline, however, they die. From this it appears that the area opaca itself contains the necessary raw materials for blood-formation and produces plasma or a plasma-like

fluid in which the haematopoietic cells are able to develop. In the explanted blood islands of amphibian embryos the blood cells also seem to develop in a plasma¹²⁵.

Haemopoiesis takes place in mammalian tissue cultures, e.g. in cultures of human foetal liver², in which it is quite active. Erythropoiesis in mammalian bone-marrow is greatly stimulated by the addition to the medium of active liver extract⁵³. Cultures of rabbit or guinea-pig bone-marrow were grown in homologous plasma to which were added glucose, a carbonate buffer, and either extract of the animal's own liver or some liver extract known to be active against pernicious anaemia. Erythropoiesis was profuse in the presence of an active liver extract which was effective in a concentration of 1:300,000; clinically inactive extracts failed to promote erythropoiesis in the cultures.

h. Connective Tissue Pigment Cells

Connective tissue pigment cells differentiate readily *in vitro*^{14,56}. Mesoderm from 3–5-day Black or White Leghorn chick embryos was explanted, no pigment cells being visible at this stage⁵⁶. After a few days they began to differentiate in the explants. When they first appeared the pigment cells were only distinguishable from fibroblasts by the presence of many colourless granules in the cytoplasm; within 10–12 hours these granules turned yellow, then brown, and at the same time long branched arms of cytoplasm were thrown out which were in active amoeboid movement. When cultures of Black Leghorn tissue were grown in medium from White Leghorn fowls, pigment-formation was unaffected.

It has been shown that the connective-tissue pigment cells are derived from the neural crest; when regions containing the presumptive neural crest were cultivated *in vitro*, pigment cells of the type described above developed in the cultures¹⁴. If the explants were taken from embryos between the late primitive streak and 20-somite stage, pigment developed only in cultures containing the neural crest; explants taken from any region lateral to the neural crest formed no pigment cells. On the other hand, many pigment cells appeared in explants of the lateral mesoderm taken from slightly older embryos in which the crest cells had already wandered into the adjacent tissue.

Interesting experiments were made with pigment cells from embryos of different breeds¹⁴. The most intense pigmentation occurred in cultures from embryos of dominant black breeds. Melanophores, however, are present in the embryos of both dominant and recessive white breeds and were able to differentiate when the tissue was explanted *in vitro*¹⁵⁴. Melanophores from recessive whites were less viable than those from dominant whites, and these again were much less viable than the melanophores of black breeds¹⁵⁴.

i. Skin

There are a few observations on the development of skin *in vitro*^{8,72,84}. When early ectoderm from either avian or mammalian embryos is cultivated, the various cell layers develop, and usually the epidermis keratinizes precociously as compared with the normal. The ectoderm may spread over the mesodermal tissue, enclosing it completely, or it may roll into an islet with the Malpighian layer outermost and the keratin in the centre⁸⁴. Histochemical studies⁸⁴ showed that eleidine and pre-eleidine are formed as in normal keratinization.

j. Hair

Hairs form *in vitro*. In explants of skin from foetal guinea-pigs hairs developed in the follicles or, if already present, continued to grow¹²⁸. In recent unpublished experiments by M. Hardy the skin of early mouse embryos in which follicles had not yet appeared was explanted *in vitro*, and both follicles and hair were formed during cultivation.

k. Thyroid

The best example of the development of a gland *in vitro* is presented by the embryonic chick thyroid^{6,7}. The thyroid rudiment, consisting of epithelial cords with no follicular structure or colloid secretion, was removed from 8-day chick embryos and cultivated *in vitro*. During the next few days follicles developed and colloid was secreted as actively as *in vivo*. The course of histogenesis was similar to that in the normal embryo and proceeded at about the same rate.

l. Pituitary

The anterior lobe of the pituitary of young rabbits has been kept in a partly differentiated state for 3–4 months by cultivation in the blood-plasma of adult rabbits. The explants seemed to retain at least part of their physiological activity, and when grown in contact with other tissues, e.g. osteogenic cells, they promoted both growth and differentiation^{31,32,126} in the adjacent culture.

m. Kidney

One of the earliest studies on histogenesis *in vitro* was made on the kidney rudiment¹¹⁷. Fragments of the metanephros of early chick embryos were explanted. Such explants are very transparent, and it was possible to observe in the living tissue the development of the glomerulus and S-shaped convoluted tubules and their union with the collecting tubules. The differentiation of blood sinuses, blood islands, and capillaries was also seen.

n. Alimentary and Respiratory Tracts

The differentiation *in vitro* of complexes consisting of the rudiments of the oesophagus, stomach, and duodenum, combined with that of the respiratory tract, has been investigated⁵⁴; the explants were obtained from 4–5-day chick embryos and were cultivated by a special perfusion technique. A complete muscular coat, showing regular peristaltic contractions, developed round the digestive tube, and the epithelium and subjacent tissue gave rise to a relatively normal mucous membrane. The bronchial tubes divided dichotomously into rather wide diverticula lined with flattened epithelium, and a series of cartilaginous nodules differentiated in the walls. At the time of explantation the mesoderm forming the wall of the oesophagus is indistinguishable from that of the bronchi, and its developmental specificity in the two organs is striking.

o. Ovary

The gonads have been cultivated by several workers^{28,34,74,75,88,109}. The ovary develops *in vitro* more readily than the testis. The foetal ovaries of rats and mice (Plate 2, Fig. 5*a*) were explanted at a stage when they consisted of a dense tissue composed of large oogonia and small somatic cells⁷⁴. After about 2 days' cultivation some of the oogonia entered the first stages of the meiotic prophase (leptotene and zygotene) while others divided by ordinary mitosis; soon afterwards structures resembling Pflüger's cords appeared, packed with oocytes and follicular cells; many mitoses were present in the single-layered germinal epithelium. After 3 days the oocytes entered the contraction phase, from which most of them had emerged by the 6th day, to pass into the pachytene phase; this was quickly followed by the diplotene. The nuclei of the oocytes entered a resting phase and some of the resting ova began to enlarge, though they never acquired a zona pellucida. Up to this stage the development of the ovary was almost normal, but after this period two important abnormalities appeared: the follicular cells failed to form mature Graafian follicles, and the ova did not move towards the interior of the organ. The ova, which grew rather more slowly than *in vivo*, attained their maximum size by about the 18th day of cultivation (Plate 2, Fig. 5*b*), and at body temperature the explant began to degenerate after 26–8 days *in vitro*. If cultivated at a subnormal temperature (34° C.), however, the cultures remained healthy for more than 100 days. The early stages of oogenesis have also been observed in explants of embryonic chick ovaries³⁴.

The effect of the gonadotropic hormone on ovarian explants from young mice has been studied²⁸. In some experiments combined cultures were made of the ovary and of the anterior lobe of the hypophysis, in others a gonadotropic agent was added to the culture medium, but no

gonadotropic effect could be demonstrated. It was suggested that the local vascular reaction produced by the hormone *in vivo* may be a very important factor in obtaining a positive effect; any vascular reaction is, of course, eliminated *in vitro*.

p. Testis

When the testes of infant mice were explanted before meiosis had begun⁷³, all stages of the meiotic prophase up to the diplotene took place normally. Differentiation stopped after about 10 days, however, and the explants reverted to an embryonic condition in which the tubules contained only supporting elements and a few large cells, presumably spermatogonia. The explants survived and grew in this simplified condition for several weeks.

Experiments have been made on the effect of cultivating fragments of testis from adult and immature rats in plasma obtained from animals of different ages^{32,33}. Explants of adult testis rapidly degenerated in all media. The most immature testes differentiated to some extent in blood-plasma from both adult and young animals, as indicated by an increase in the interstitial connective tissue, the formation of spermatids, and the widening of the tubules with the appearance of lumina. The more mature testes showed appreciably less development in any medium.

q. Eye

Even such a complex structure as the eye possesses a remarkable capacity for self-differentiation *in vitro*^{13,130}. An eye rudiment of a 64–72-hour chick embryo (Plate 2, Fig. 7*a*) consists of an undifferentiated multi-layered retina covered externally by the cubical epithelium, usually unpigmented, of the tapetum, and a simple vesicular lens. If such a rudiment is isolated and grown in culture, the retina forms all the different zones of the pars optica (Plate 2, Fig. 7*b*), viz. cones, inner and outer plexiform layers, ganglion cells, and nerve fibre layer; the pars ciliaris retinae differentiates, the tapetum becomes densely pigmented, and the vesicular lens forms fibres. Differentiation proceeds at almost the normal rate, although the explants attain a maximum size of only 1–2 mm.; thus inhibition of normal cellular multiplication is not necessarily correlated with inhibition of normal differentiation. The shape of the eyeball is hopelessly distorted, probably owing to the absence of the firm, supporting sclerotic.

Similar results have been obtained with the foetal rat eye^{143,144}. Eyes were removed from 14-day rat embryos, at which stage there is very little histological differentiation; after 15 days' cultivation they had developed a pigmented ciliary body and a retina with ganglion cell layer, inner fibre layer, and young rods. As in the chick eyes differentia-

tion proceeded at a nearly normal rate, although growth was even more restricted than in the avian eye rudiment.

Explants of the chicken eye rudiment have been used to investigate the induction and regeneration of the lens^{147,148}. It was shown that the optic vesicle of 2–5-day chick embryos is able to induce a lens in non-presumptive ectoderm; at first all the ectoderm has this capacity for lens formation, but later in development only the head ectoderm and finally only the presumptive corneal region respond. Up to the 4th day of embryonic life the explanted eye cup can regenerate a lens from the border of the iris, and in explants of 3-day eyes a lens can be regenerated even from the pigment layer.

r. Ear

The avian otocyst differentiates in culture as readily as the eye¹⁶. When removed from 3-day embryos it formed all the epithelial constituents of the fully developed auditory labyrinth, and regions identical in histological structure with the normal ductus and saccus endolymphaticus, sensory areas including Corti's organ, and the tegmentum vasculosum, developed *in vitro*. Although the principal structures appeared in approximately the same relative positions as in the normal embryonic labyrinth, the explants showed only slight indications of a gross anatomical differentiation, and tended to retain the primitive vesicular form. As in the eye cultures, the rate of differentiation was almost normal, while the rate of growth was enormously retarded.

s. Nervous Tissue

Finally we come to the differentiation *in vitro* of nervous tissue. It is interesting to recall that the tissue-culture technique was originally devised by Ross Harrison for his classical experiments on nerve outgrowth. Harrison⁴⁵ explanted a piece of embryonic amphibian nerve cord in a hanging drop of lymph; nerve fibres grew out from the cord into the surrounding medium, thus settling the controversy as to whether the axon was formed by an outgrowth from the nerve cell or was secreted by the sheath cells.

Since Harrison's publications appeared much work has been done on the cultivation of nervous tissue from many different species. As G. Levi points out, however, in his comprehensive review of the subject⁵⁹, it is unwise to draw conclusions about the normal development of nervous tissue *in vivo* from observations made *in vitro*, since the differentiation of nervous tissue in culture soon becomes atypical; on the other hand, cultures of nerve tissue are valuable for studying the biological properties of the nerve elements.

When a fragment of embryonic nervous tissue is explanted *in vitro*, neurites soon emerge from the explant, elongate, and often branch; the

outgrowth is largely effected by amoeboid activity at the tips of the fibres. Neuroblasts and supporting elements also migrate from the explant, and under suitable cultural conditions a complex network of cells and fibres is formed which may survive for weeks or even months^{62,82}. Cultures of human foetal brain have been kept in a healthy differentiated state for more than 140 days⁵⁰. Ependymal cells from early human embryos also remained differentiated and functional *in vitro* for long periods⁴⁹; they formed vesicles lined by ciliated columnar epithelium, the cilia of which were beating actively 83 days after explantation.

Adult human sympathetic ganglion cells survived and multiplied *in vitro* for several months⁹⁴ and presented features which *in vivo* characterize foetal and regenerating ganglion cells. Many ganglion cells (Plate 2, Fig. 8) migrated from the explant for a distance of several millimetres, and some underwent mitotic division; they did not become organized into a tissue or form synaptic relationships. Ganglion cells in the explant frequently produced branching nerve fibres which extended for about 2 mm. into the medium and survived for months; they were often closely invested by Schwann cells, which migrated and divided as in normal development.

The examples of nerve growth *in vitro* quoted above do not refer to a progressive differentiation *in vitro* equivalent to that which takes place in normal embryonic life. Normal differentiation may occur in cultures, however. Thus, in explants of the medulla of the early chick embryo the motor neuroblasts develop from an apolar to a bipolar condition¹³², and ganglion cells change from a bipolar to a unipolar state¹³¹. There is evidence that the normal development of nerve tissue is promoted by and in some cases may entirely depend upon association with other tissues. For example, it has been shown¹⁴² that the neural canal from chick embryos of less than 32 hours' incubation does not differentiate *in vitro* unless it is accompanied by the tissues which normally surround it; on the other hand, similar explants from 32-6-hour chicks are able to differentiate in complete isolation.

Probably the most complete example of neuro-histogenesis *in vitro* is seen in the development of the autonomic nerve plexus in compound explants of the oesophagus, stomach, and duodenum⁵⁵. Here again the striking normality of the developmental process was probably due to the fact that the neurogenic elements were in the normal morphological surroundings. In these experiments the cultures were prepared in the same way as those described on p. 436, and the development of the nerve plexus could be studied from day to day in the living cultures as well as in fixed material. The neuroblasts, which at first resembled mesenchyme cells, remained in connexion with each other throughout their differentiation, their cytoplasmic processes developing into nerve fibres. Later, the scattered network of neuroblasts congregated into

dense, compact groups like those which characterize the normal peripheral autonomic nervous system.

iv. FUTURE APPLICATIONS

Examples of the differentiation *in vitro* of a wide range of tissues have been described in this chapter, but although this type of tissue culture has been applied to many different problems, the method could be exploited far more extensively than has been done hitherto.

In particular, it is astonishing that so little work has been done on the metabolism of differentiated tissues in culture. For example, while there is a large literature on the nutritional requirements of unorganized tissue *in vitro*, hardly anything is known about the special nutritional needs of the various types of differentiated tissue; yet it is a matter of common experience that such tissues are usually very sensitive to alterations in the culture medium^{23,32,83,85}. The fact that so many kinds of tissue can be grown in a state of nearly normal differentiation when completely isolated from the complicating influences of the body as a whole would seem to offer a unique opportunity for investigating their physiological requirements with a precision that could hardly be achieved by any other method. Differentiated tissue cultures bear a much closer relation to the tissues of the normal body than do unorganized cultures, although the latter provide very valuable material for studying the general problems of growth, and it seems likely that conclusions drawn from observations on a histologically normal tissue *in vitro* would largely apply to the same tissue in the intact animal.

In the writer's experience the chief difficulty in growing organized tissue lies not in the culture technique, which is relatively simple, but in preparing and handling the tissue; this may require considerable manipulative skill, since the explants must often be obtained from early embryos, and their dissection must be accomplished with minimal damage to the cells. Collaboration between a biochemist and someone who is not only experienced in the technique of growing organized tissues but also possesses an intimate knowledge of histology and morphogenesis might yield results of the greatest interest and importance, and it is to be hoped that work along these lines will soon be undertaken.

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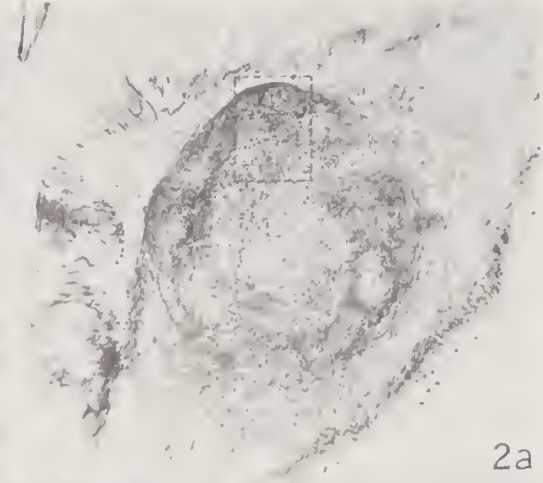
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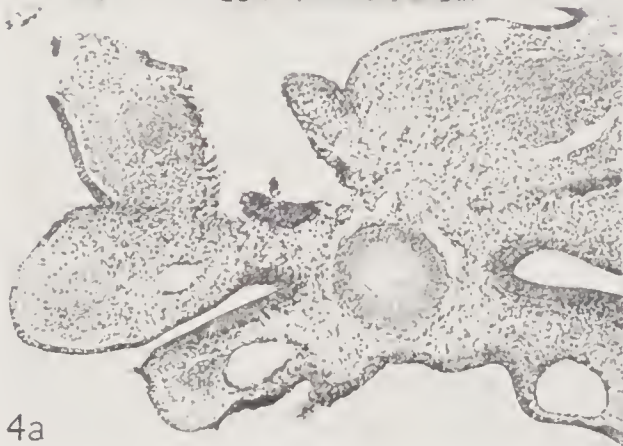
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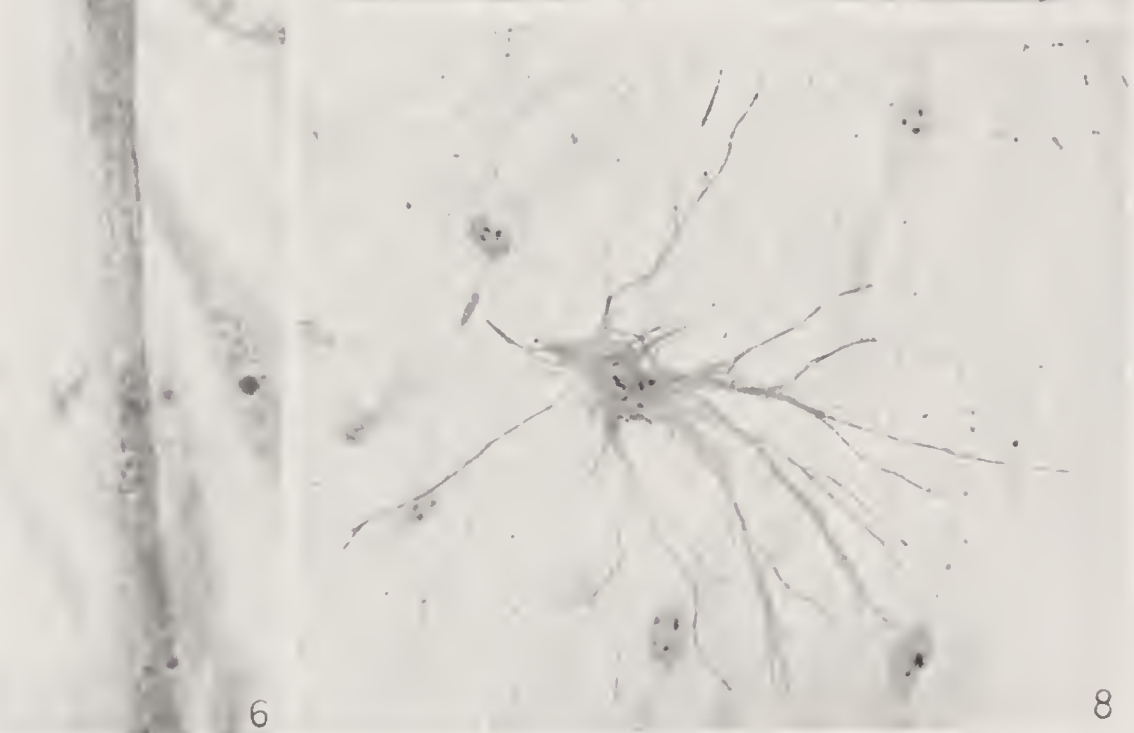
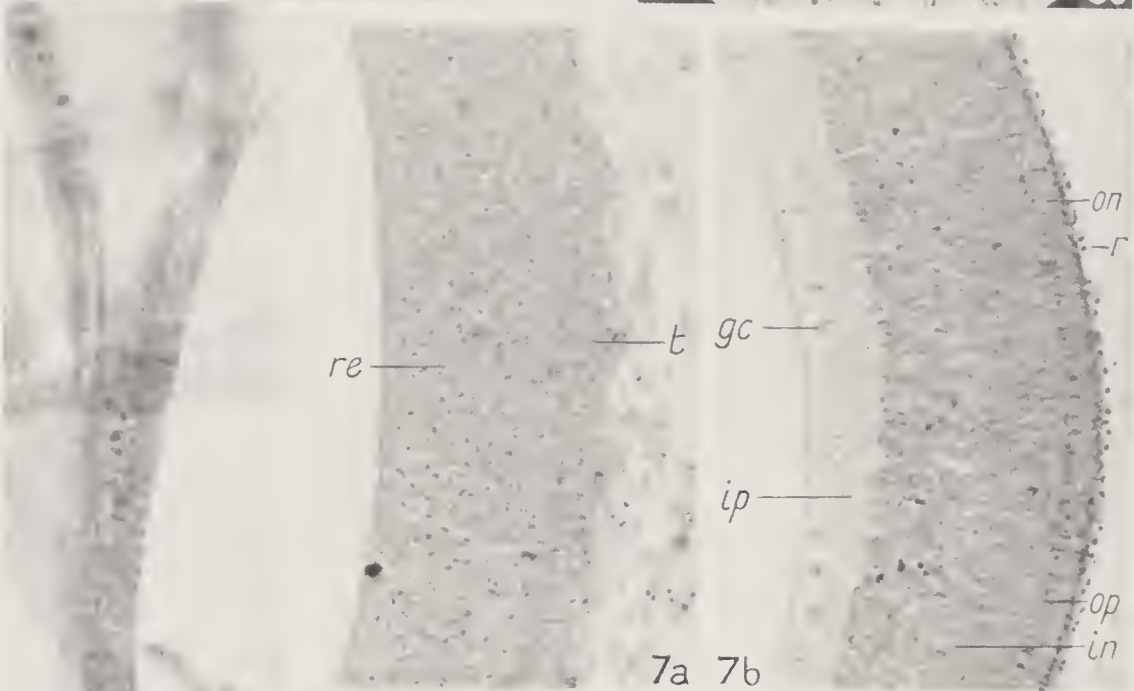
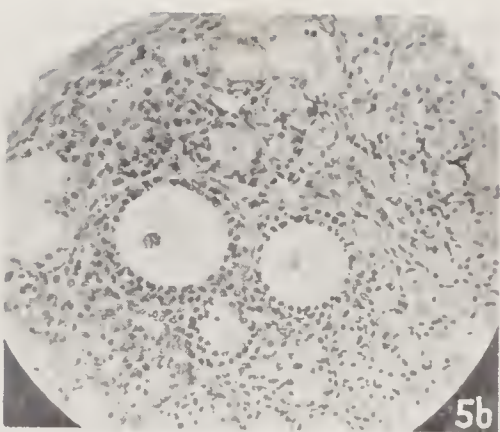
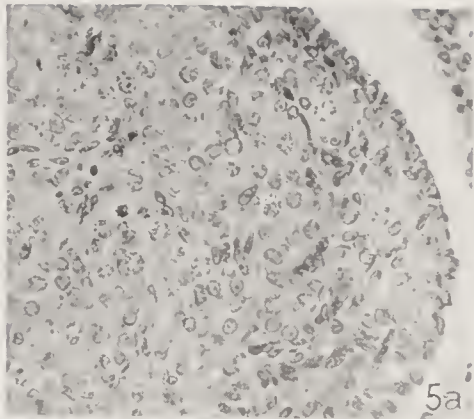
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DESCRIPTION OF PLATES

PLATE 1

FIG. 1. Intercellular fibres formed in a culture of perivascular tissue from a 9-day chick embryo. (After Momigliano-Levi⁸⁷.)

FIG. 2. *a*. Culture of endosteum from the tibia of a 21-day chick embryo, 8 days after the removal of the original bone explant (sec p. 426). New bone is being formed in the undisturbed interior of the culture (section parallel to the plane of the cover-slip).

b. The field marked in 2*a* seen under higher magnification (culture by H. B. Fell).

FIG. 3. *a*. Ten-day culture of periosteum stripped from the unossified tibia of a 6-day chick embryo. A plate of bone has developed *in vitro* (section at right angles to the plane of the cover-slip).

b. The field marked in 3*a* seen under higher magnification (culture by H. B. Fell).

FIG. 4. *a*. Frontal section through the mandible of a 3-day chick embryo. On the left (*ex*) the chondrogenic area of the undifferentiated mesoderm has been excised and explanted *in vitro*.

b. The explant removed from the mandible shown in Fig. 4*a* after 8 days' cultivation *in vitro*. A rod of hyaline cartilage has developed in the culture. (After Jacobson and Fell⁵², by courtesy of the *Quarterly Journal of Microscopical Science*.)

PLATE 2

FIG. 5. *a*. Normal (control) ovary from a 19–20-day rat embryo.

b. Opposite ovary from the same embryo after 21 days' cultivation *in vitro*. Typical Graafian follicles have developed during cultivation, and the nuclei of the oocytes have entered the resting stage. (After Martinovitch,⁷⁴ by courtesy of the Royal Society.)

FIG. 6. Cross-striated muscle ribbon from a 20-day culture of adult human muscle. (After Pogogeff and Murray¹¹¹, by courtesy of the Wistar Institute Press.)

FIG. 7. *a*. Normal (control) retina of a 3-day chick embryo. At this stage the retina (*re*) consists of an undifferentiated many-layered epithelium, covered externally by the cubical epithelium of the tapetum (*t*).

b. The retina from the opposite eye of the same embryo, after 9 days' cultivation *in vitro*. The rods (*r*), outer nuclear layer (*on*), outer plexiform layer (*op*), inner nuclear layer (*in*), inner plexiform layer (*ip*), and ganglion cell layer (*gc*) have developed *in vitro* (culture by T. S. P. Strangeways and H. B. Fell).

FIG. 8. Stellate ganglion cell in a 42-day culture of an adult human sympathetic ganglion. (*ax* = axon.) (After Murray and Stout⁹⁴, by courtesy of the Wistar Institute Press.)

CHAPTER XI

SOME ASPECTS OF EVOLUTIONARY CYTOLOGY

By E. N. WILLMER

i. INTRODUCTION

HISTOLOGY and cytology are subjects which have been largely stimulated by the applications which they have to medicine. For this reason mammalian and particularly human structures tend to have been studied as ends in themselves. This very specialization on the structures of higher animals and man may, however, be the cause of certain misunderstandings and false theories which could never have arisen if a broader base had been adopted for the histological edifice. From this point of view the human body may perhaps be compared to a city like London. A stranger from another country deposited in London and having no knowledge of its history would find it difficult to understand the significance to be attached to the name of a street called Poultry, or to men wearing long black gowns and white wigs in some picturesque but not very ecclesiastical-looking buildings called the Inner Temple. Yet all these things make sense if seen in relation to their place in the history of the great city. They are in a sense natural evolutionary products of the growth of the city from its early beginnings, just as in the human body the ear, for example, is what it is and where it is because it has evolved from earlier structures, such as the lateral line, the spiracle, and the bones which formed part of the suspension of the jaw in animals lower in the evolutionary hierarchy.

On the large scale, all this is very obvious and well recognized but, curiously enough, the evolutionary approach has been far less in evidence in studies of cytological and histological material. For the solution of certain problems, however, a study of both ontogeny and phylogeny may have very significant contributions to make. Animals and plants have contrived such extraordinary and apparently unlikely adaptations, both structural and functional, that one tends to think that Nature can produce what she wants, wherever and whenever she wants it. Even if this were true, it would obviously be much easier for her to produce her structures in some places than in others; for example, she apparently found it easier to put the ears of mammals in the head, but in grasshoppers the materials which could most easily be adapted to the function of hearing were more readily available in the legs, and so the grasshopper hears by means of ears in its legs. In general, it may be concluded that an organ is what it is and where it is because of its previous history; so, in particular, a cell behaves according to its previous

history, where this means not only its embryological history but, more fundamentally, its evolutionary history. A few examples may emphasize the significance of this approach to cytological problems.

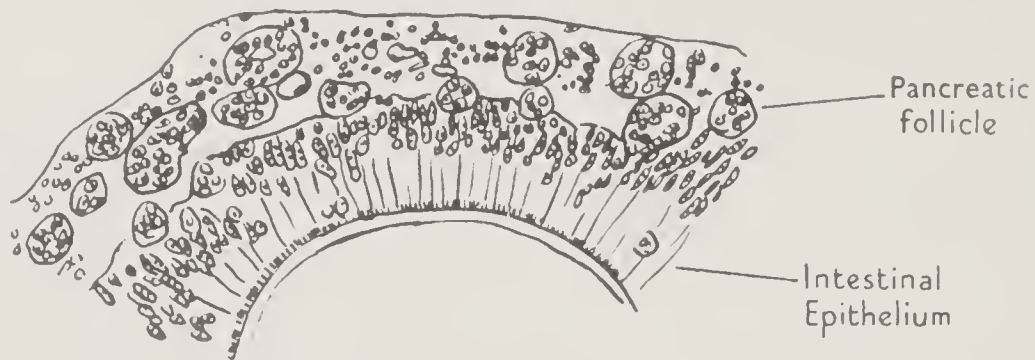
ii. EXAMPLES OF CYTOLOGICAL EVOLUTION

a. *Cells of the Pancreas*

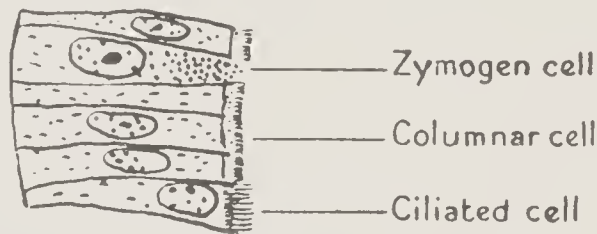
It is common knowledge that the islets of Langerhans are specialized patches of tissue in the pancreas, with the curious property of responding to increase in the glucose content of the blood which bathes them by liberating insulin into the blood. On the face of it, this looks like an act of God, and students of human physiology have to remember it. Nevertheless, embryology shows clearly that the islets develop as abortive outgrowths of the ducts of the pancreas and that they are thus composed of modified duct cells. Moreover, the ducts are outgrowths of the wall of the duodenum, and from an evolutionary point of view may be regarded as a specialization of the glands of the duodenal mucosa. The origin of these glands themselves is a matter worthy of further investigation and, as will be shown later, may perhaps be found in the vagaries of another system, altogether different from the alimentary canal. The duodenal mucosa is a structure specialized for absorption as well as secretion, and there would be obvious advantage to be gained if the mucosa, at the same time as it passed glucose from the gut into the blood, also liberated the substance, insulin, which made the glucose available to the tissues. It is therefore interesting to find that in the ammocoete larva of the lamprey, an organism which may be regarded as an extremely primitive vertebrate and which does not possess a true pancreas, there are to be found certain islands of cells, called the follicles of Langerhans (Boenig¹⁶), situated in the submucosa of the alimentary canal at the junction between the fore-gut and mid-gut. The cells probably arise by budding off from the duodenal mucosa and sinking into the submucous connective tissue (Text-fig. 1). They then lose their connexion with the intestine and become very well supplied with blood-vessels. Their destruction causes a significant rise in the level of sugar in the blood. In all probability, therefore, these cells are endocrine in function, secreting an insulin-like substance, and they can probably be regarded as homologous with the islets of Langerhans in higher animals (Barrington¹⁰). The intestinal epithelium itself is interesting in the same region, for it contains cells unlike those of the rest of the alimentary canal in that they contain more zymogen granules, have basophil cytoplasm, and nuclei with pronounced nucleoli (Text-fig. 2). These cells produce a trypsin-like enzyme and clearly suggest comparisons with the exocrine portion of the pancreas (Barrington^{10,12}). In the ammocoete, therefore, there seem to be present in the intestinal epithelium and its

submucous connective tissue all the necessary ingredients for the production of the true pancreas of higher forms, an organ which then develops in the form of outgrowths from the duodenal mucosa.

Moreover, the propensity of the cells of the follicles of Langerhans to



TEXT-FIG. 1. Section through the region of the anterior intestine in the ammocoete larva of *Petromyzon*, showing the position of the follicles of Langerhans. (From Boenig¹⁶.)



TEXT-FIG. 2. Cells from the epithelium of the intestine of *Petromyzon*, showing the three types of cell. (From Barrington¹⁶.)

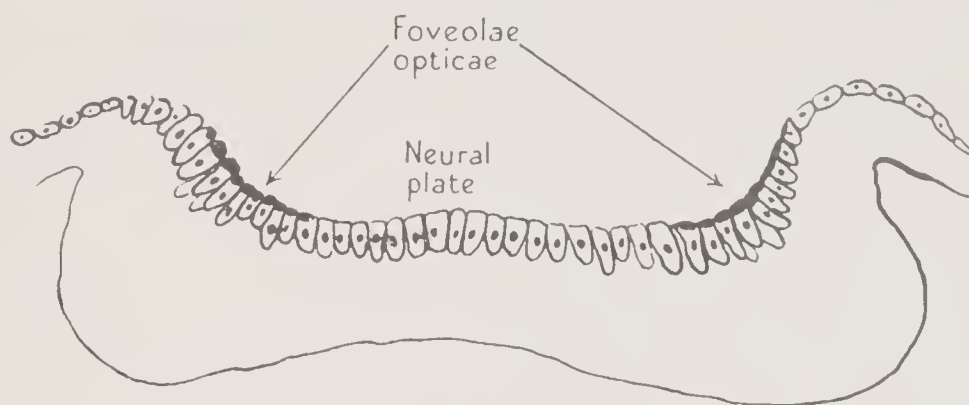
leave the epithelium proper and to sink into the submucous layers must have some significance. A similar outgrowth of cells leads to the formation of the glands of Brunner in higher mammals and man, though in these glands the connexion with the gut epithelium is not entirely severed. The glands of the oesophagus in higher animals also show similar characteristics. If, then, the glands of Brunner and the cells of the islands of Langerhans are both derived in this manner from cells related to those in the primitive follicles of Langerhans, further comparisons between the two may bring to light new features in their physiology. On the same lines, the control of the secretion of the pancreas by means of hormones, secretin, and pancreozymin becomes more intelligible when the probability of a common origin for the exocrine cells of the pancreas and the epithelium of the mucosa of the duodenum is kept in mind.

b. Cells of the Retina

Another group of tissues, upon whose interrelationships the evolutionary approach may give valuable information, includes the retina and its associated structures. The retina of the human eye presents an extremely complicated histological picture; it is apparently inside out and possesses two types of receptor cell, one of which is concerned with

vision in ordinary daylight and the other functions at very low brightness levels, thus providing for day and night vision respectively. Such is the impression created in the mind by the average description of the eye in physiological text-books. But a study of the evolution of the eye and of its embryology opens up numerous fascinating relationships between the cells concerned and suggests endless problems for research.

As Text-fig. 3 shows, the eyes develop from groups of cells of the neuro-epithelium, which in the embryo amphibian appear as two slightly



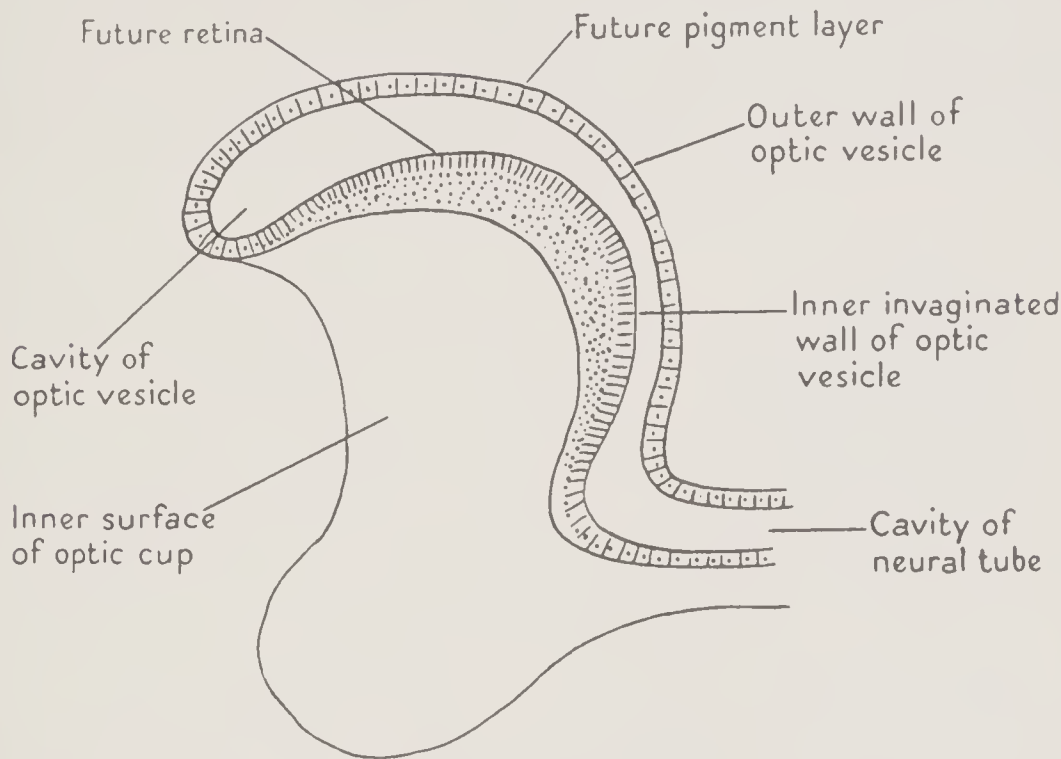
TEXT-FIG. 3. The foveolae opticae in the developing frog.

pigmented structures, the foveolae opticae. Unfortunately there appear to be no animals which show the all-important stages by which the pattern of the vertebrate eye was first evolved, and various theories have been put forward to account for the exact manner in which the retina attains its inverted form. There is, however, general agreement that its cells are derived from the neuro-epithelium, and in particular from certain cells in it which, in earlier forms, were presumably sensitive to light. The formation of melanin pigment was also a characteristic of these cells, and may indeed have been partly responsible for their sensitivity to light. The cells of the neuro-epithelium, of course, mainly give rise to the neural tube and hence to the nervous system in general, together with its lining of ciliated ependymal cells. Such ependymal cells are visible in the optic nerve of lampreys and, as will be shown, cells corresponding to the ependymal cells of the neural tube play a prominent part in the development of the retina, ciliary processes, and iris. The cells of the primitive neuro-epithelial plate, which lie lateral to the foveolae opticae, as they fold inwards in the formation of the neural tube, become the last to be included in the tube, or they may just escape inclusion and then contribute to the 'neural crest' tissue. Thus the cells of the foveolae opticae lie in the neuro-epithelium in a position which is intermediate between the neural plate proper and the presumptive neural crest. They may, in consequence, be expected to display certain affinities to the cells in both groups. Embryologically there seems to be a gradient of neural properties from the centre of the floor of the neural plate outwards towards the general ectoderm of the body, and

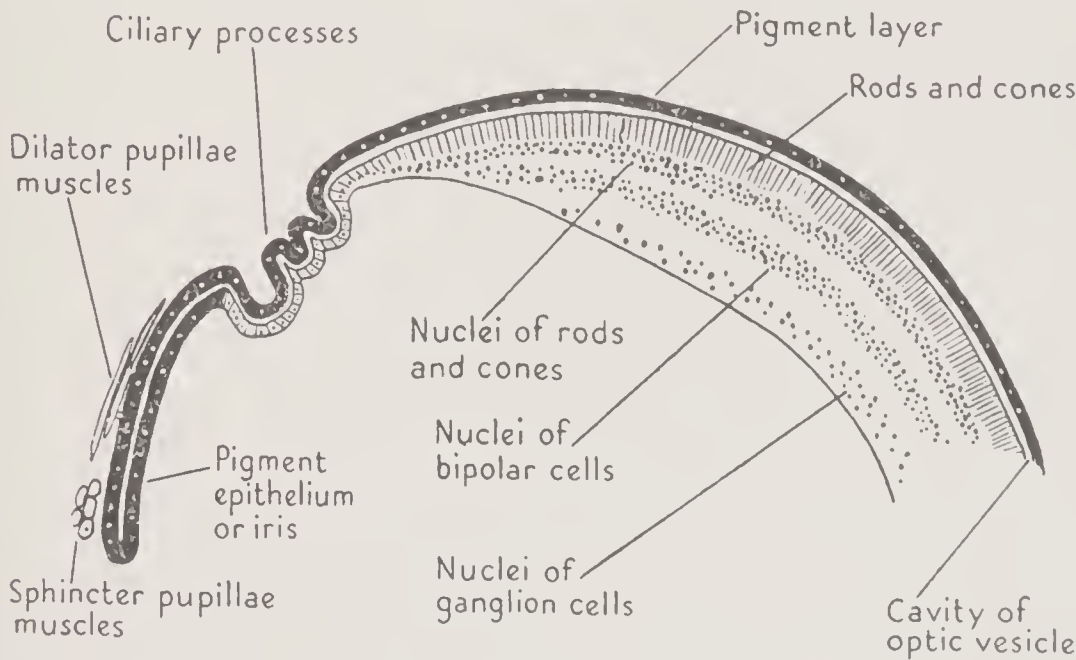
the neural crest lies towards the outside limit of this gradient system. Now if the formation of melanin, for example, is considered together with another process which is probably akin to melanin formation and which certainly involves a special form of tyrosine metabolism, namely, adrenaline formation, it will be found that these activities are normally restricted in the body to the ectodermal epithelium and the neuro-epithelium. They are most developed in the intermediate zone just described, namely, the neural crest and its derivatives. It has been shown, both in amphibia (Du Shane²⁸) and in birds (Dorris^{26,27}; Eastlick²⁹), that this tissue probably gives rise to all the melanophores of the animal, and it is generally agreed to be the seat of origin of the chromaffin tissue, adrenal medulla cells, and the cells of the sympathetic ganglia. The cells belonging to the other ganglia of the body emerge either from neural crest tissue or from the upper parts of the neural tube itself. The pia mater is also a neural crest derivative, so that the pigmented structures associated with this membrane thus fall into line; these include the pigment cells of the choroid of the eye, since the latter probably arises by modification of the cells which elsewhere form the pia mater. The formation of the pigment may be even more restricted to this zone than the above account suggests, for if the hypothesis (Billingham¹⁵) that the dendritic cells of the skin provide the melanin for the normal epithelial cells of the epidermis is substantiated, and it seems likely that the dendritic cells have their origin in the neural crest, then it would appear that only neural crest cells and their immediate neighbours might have this power, for the pigment which occurs in nerve cells proper is very restricted and may have some special cause of its own. The pigment layer of the retina, including, of course, that of the iris, clearly falls within the same group, as can be seen by a study of Text-figs. 4 and 5, which show the relationship between the different parts of the eye as it develops by the invagination of the optic vesicle. There is a continuous epithelial layer from the lining of the neural tube, the future endoderm, via the pigment epithelium of the retina, the pigmented epithelium of the ciliary processes, and the pigment layer of the iris, which doubles back on itself, loses its pigment as it again covers the ciliary processes, and finally becomes the layer of cells which are converted into rods and cones. Thus the cells of the neural crest and its immediate neighbours seem to have 'made a corner' in a special form of tyrosine metabolism, which may culminate either in the formation of melanin or of adrenaline.

An interesting feature of the melanin-containing cells is presented by a comparison between the pigmented cells of the choroid and those of the pigment layer of the retina (Text-fig. 6). The latter are purely epithelial; all the cells are adherent to each other and closely packed into a hexagonal pattern. The cells of the choroid, on the other hand,

are amoeboid and irregular in shape; they occur as isolated cells and have no tendency to adhere to each other. The melanophores of frogs and birds and the dendritic cells of the skin behave like the choroid



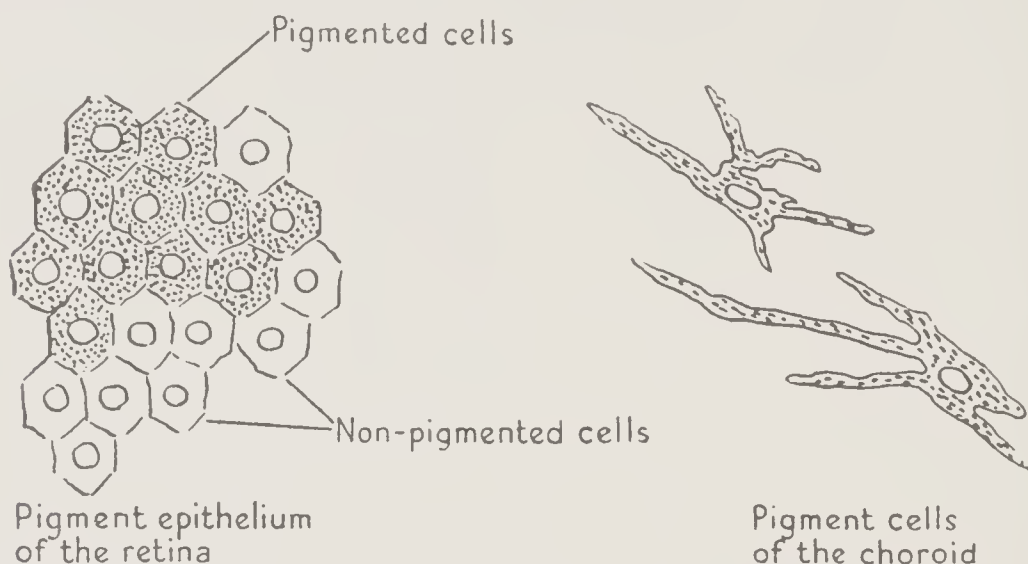
TEXT-FIG. 4. Diagram showing the origin and development of the retina from the optic vesicle.



TEXT-FIG. 5. The cell layers in the developing retina and associated structures.

cells. The nature of the relationship between these two cell-types is clearly a problem worthy of investigation. The pigment epithelium in the eyes of animals like the sheep may throw light on these problems because it is often found to be pigmented only in patches and, as already indicated, the ciliary processes are covered by one pigmented and one non-pigmented layer. Moreover, some of the amoeboid pigment cells

in the substance of the iris can be shown to be directly derived from the adjacent pigment epithelium (Lewis⁴⁴). One or two other types of cellular behaviour in the eye would become more intelligible if the neuro-epithelium had a dual character, and the matter will be taken up again later in another connexion. Meanwhile, it may be supposed that when the pigmented neural crest cells leave the epithelium and sink into the underlying mesenchymal tissues they acquire the amoeboid



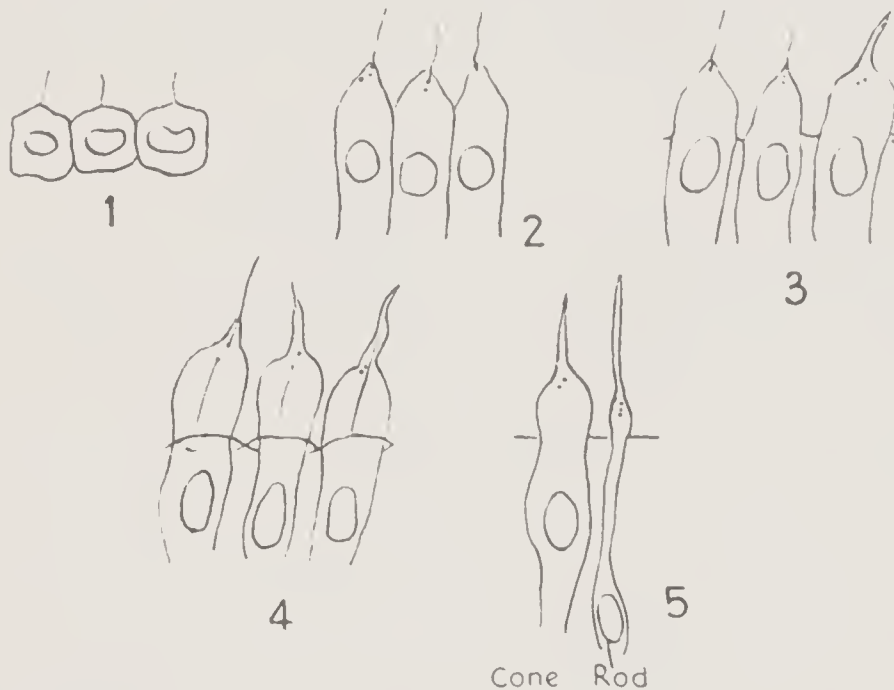
TEXT-FIG. 6. Pigment cells from the pigment layer of the retina and from the choroid.

form seen in the cells of the choroid, and the further problem is raised as to the nature of the change which has occurred to cause certain cells to leave the surface and undergo this alteration of form and behaviour. The same problem arises in connexion with the iris.

The derivation of the optic vesicle from the neural tube and the homology between the rod and cone cells and the cells which line the neural tube is emphasized, even in the human embryo (Mann⁴⁷), by the fact that, when the rods and cones begin to develop and to differentiate histologically, the first structures which appear are flagella, and the form of the cell is essentially that of the ependymal cell. It is only later that the flagella are modified into the outer segments of the rods and cones (Text-fig. 7).

Another important feature of this layer of cells which lines the original optic vesicle is that it gives rise to cells which are light-sensitive, not so much in the sense that they set up nerve impulses in the sensory nerve fibres connected with them, but in that they will respond to light by actual movement themselves. This response is sometimes direct and sometimes secondary. In the latter case it is initiated by other more sensitive cells of the same group, e.g. the cells of the retina proper, and then relayed to the cells in question, the effector cells, either by hormones or by nerves. A clear example of this is found in the sphincter and dilator pupillae muscles, for these are direct derivatives of the epithelium which lies along the posterior surface of the iris, where it

generally becomes heavily pigmented (see Text-fig. 5). In some animals they are true myo-epithelial cells in which the contractile portion forms an inner process of an otherwise typical columnar epithelial cell. In elasmobranch fishes these muscles respond directly to light, i.e. they are independent effectors (Walls⁶¹). The sphincter contracts and the dilator relaxes. In higher vertebrates the same effects are produced by light, but through the intervention of either nerves or hormones or both.

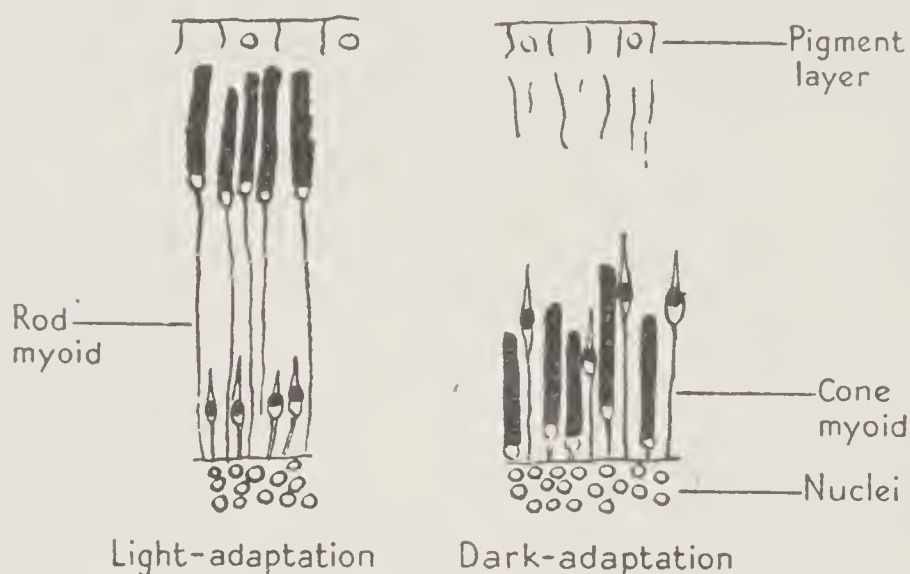


TEXT-FIG. 7. Stages in the development of the rods and cones in the human eye.

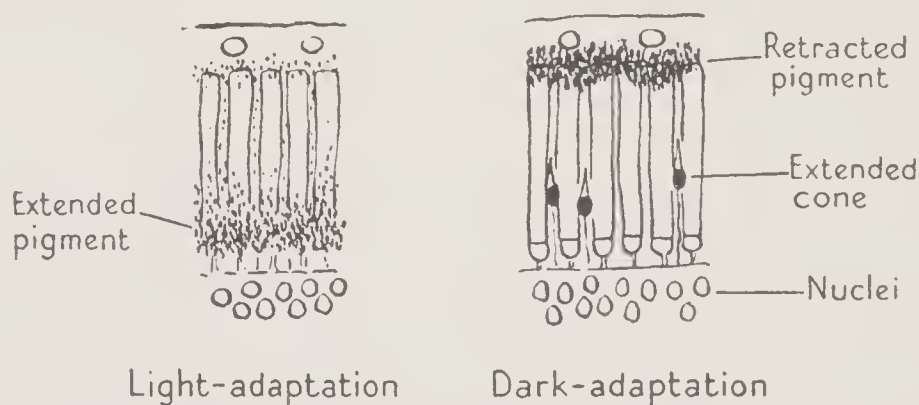
Another example of this mechanical response to light is seen in the rods and cones themselves. In many fish (*Ameiurus*, for example), when light shines on the eye it causes the contractile portions of the inner segments of the cones, the myoids, to contract and those of the rods to extend (Text-fig. 8) (Detwiler²⁵). In fishes it is probable that such reactions are essentially direct photomechanical changes, but they are often controlled secondarily by nerves or hormones. In the frog there is a similar response by the cones, but it depends on the action of adrenaline. In higher vertebrates such responses only occur sporadically, and in man there is not much evidence to suggest that the photoreceptors have any power of movement at all.

A third example of the response to light on the part of the cells of the neuro-epithelial layer in the eye is the movement of pigment granules in the cells of the pigment layer. In light, the pigment tends to extend forwards among the processes of the rods and cones, in darkness it retracts (Text-fig. 9). Again these movements are much more prominent in lower animals, and there is little evidence for their existence in man. They, too, may be controlled by hormones. In the frog, adrenalin causes the expansion of the pigment, and the movement will not occur naturally if the adrenal glands have been removed (Hasama⁴¹). This

action in the frog is interesting in another way, for it is exactly the opposite of the reaction of the melanophores elsewhere in the body to adrenalin, for these latter generally contract to this hormone and expand to the hormone produced from the pars intermedia of the pituitary



TEXT-FIG. 8. Diagram showing the position of the rods and cones in the retina of *Ameiurus* under conditions of light- and dark-adaptation.



TEXT-FIG. 9. Diagram showing the position of the pigment in the retina of the frog under conditions of light- and dark-adaptation.

body. This again raises the question of the relationship between these two types of melanin-containing cells, and also the question raised earlier as to whether the pigment epithelium contains actually two types of cells whose functions are fundamentally opposed to each other, one type responding to adrenalin while the other responds to acetyl choline, as in the two types of pupillary muscles. The differential response to light on the part of rods and cones might then be explicable on the same lines. The neural crest cells clearly show this duality, in that there arise from this layer the cells of the sympathetic and parasympathetic ganglia. Thus there is seen to be a further parallelism between the neural crest cells and the cells found in the eye.

In frogs and birds it has been shown that the neural crest is the tissue from which arise not only the melanophores but also the lipophores, which are wandering cells containing carotenoid pigments, and which

show many characteristics similar to those of the melanophores. There seem to be two main groups, the yellowish xanthophores containing chiefly xanthophyll, and the erythrophores containing astaxanthine, a red pigment (Parker⁵⁴). Many fish also have iridophores, which are cells of the same general type and are probably derived from the same source. They contain crystals of guanine and have the property of reflecting light. Now the eye is, as we have seen, rich in melanin-containing cells, both in the choroid and in the pigment epithelium; xanthophyll and astaxanthine have both been extracted from the oil globules in the cones of the eyes of birds, and in man the macular pigment appears to be xanthophyll (Wald⁶⁰). Moreover, visual purple is a combination of a carotenoid pigment and a protein. Finally, in many animals there is a tapetum or reflecting layer which may develop either from the pigment epithelium or from the choroid. In many cases this reflecting surface is provided by guanine crystals in the cells (Walls⁶¹).

It is true that carotenoids, melanin and guanine, are of wide distribution in the animal kingdom, but in studying the eye it is well to bear in mind these similarities and possible relationships between the retinal cells, pigment cells, and the other derivatives of the closely related neural crest and neural tissues. The rods and cones, for example, behave as though they belonged to two different but adjacent branches on the tree of cellular evolution. They seem to have diverged from a common stock in much the same antagonistic or reciprocal manner as have the sphincter and dilator pupillae muscles or, farther afield, the sympathetic and parasympathetic nerve cells. In theories of colour vision, for example, it may be well to remember the possible parallelism between the responses of melanophores, xanthophores, and erythrophores, and the reactions of rods and cones. In birds it is probable that colour vision actually does depend on xanthophyll and astaxanthine and perhaps other colour filters in the form of the coloured oil globules in the cones. So far, these substances have not been detected in a corresponding position in the cones of man, but, as already mentioned, xanthophyll is abundant in the cone-rich part of the retina as the macular pigment.

In studying problems of human vision, therefore, there are many and various features of the eye, and of the optical system generally, for which a study of the development of the machinery and of its evolution in lower animals can be of great assistance. Moreover, it is clearly important to consider the family relationships between the different groups of cells, and the peculiar metabolic as well as structural characteristics which they inherit.

c. Cells of the Urinogenital Systems

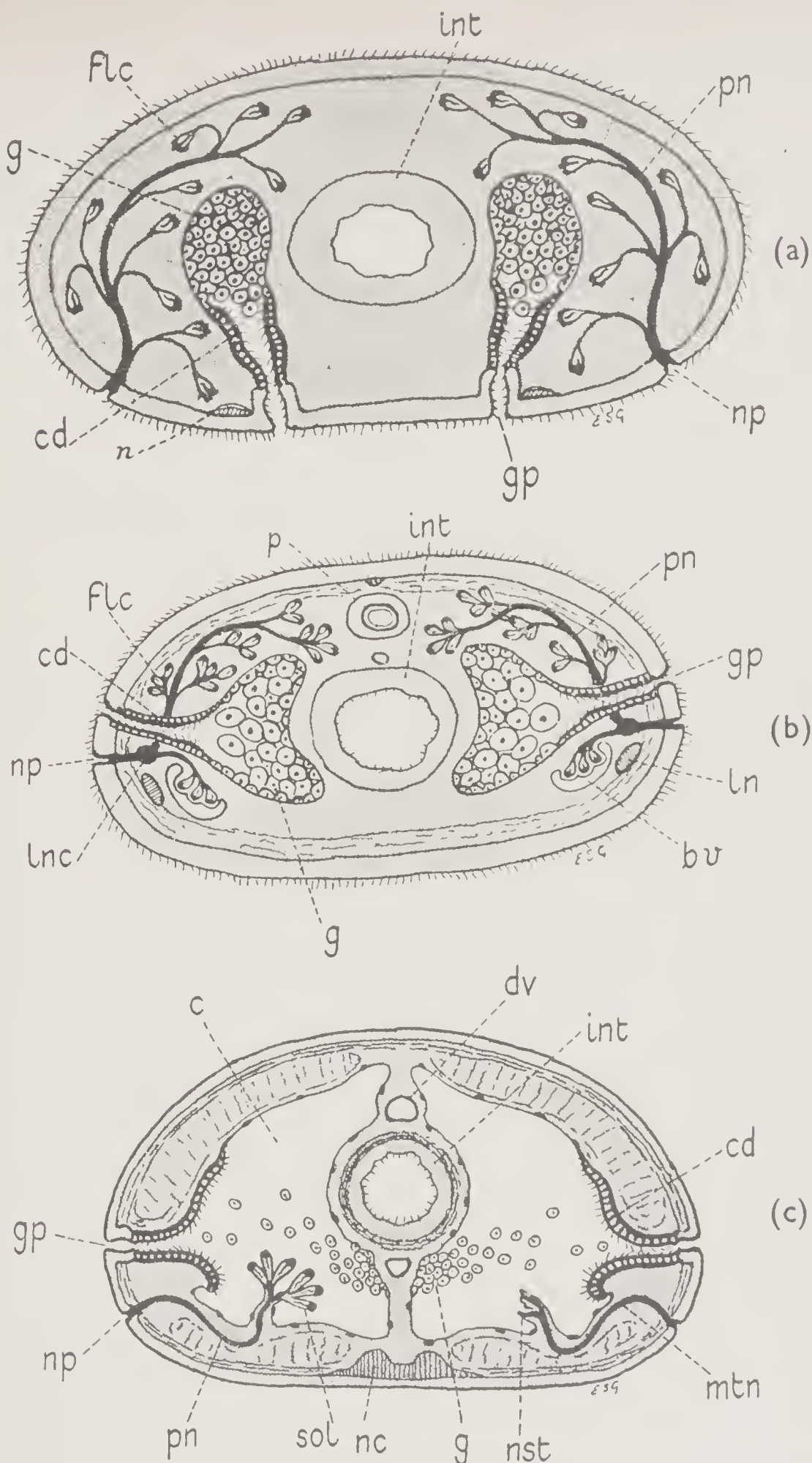
A third system of cells in the study of which the evolutionary approach may be very profitable is the urinogenital system and its associated

structures, which include the adrenal cortex and probably several other tissues besides.

In 1903 Gaskell³¹, in his now well-known discussions on the origin of vertebrates, put forward some extremely interesting arguments concerning the development and evolutionary history of the urinogenital system. Many of these arguments have been substantiated by more recently acquired knowledge, and many more are relevant to a proper interpretation of this complex system. The present discussion follows along lines which Gaskell himself actually suggested or which he clearly had in mind.

In order to study the urinogenital and related systems in higher animals it is important first to consider the corresponding systems in those invertebrates which may be related to the ancestors of the vertebrates, and then to compare the system as it is found in the early vertebrates. In the main, there are four groups of animals which are particularly important. The ammocoete larva of the cyclostome probably presents something approaching the simple vertebrate form. Secondly, *Amphioxus*, particularly in its larval form, may represent the chordate, pre-vertebrate stage, though it is necessary to remember that in this, as in other examples, the animal is, in all probability, not on the main line of descent but on a more or less specialized side-branch, and may lie in a backwater of the evolutionary stream. The third animal of interest is *Peripatus*, half worm, half arthropod; for in this animal the excretory system bears extraordinary similarity to the system in primitive vertebrates, particularly in the manner of its development. But again, *Peripatus* is unlikely to be on the direct line of descent. Finally, there are, of course, the annelid and more primitive worms to be considered, and these primitive worms will provide a suitable starting-point. Goodrich³⁴, shortly before his death, summarized much of his life's work in the form of a detailed analysis of the urinogenital systems of all the relevant animals, and his work will be freely quoted.

In primitive forms, as in the Platyhelminth, Nemertine, and Annelid worms (Text-fig. 10), there are clearly present two types of structure which seem to be completely independent. These structures are (1) the nephridia and (2) the genital sacs with their coelomoducts leading to the exterior. Typical nephridia either develop as ingrowths from the epidermis and end blindly in 'flame cells' (solenocytes), or they may arise from certain cells (nephridioblasts) which leave the epidermis at an early stage in development and then form blind tubes precisely similar to those produced by direct invagination, and these tubes open later on to the surface of the body (Text-fig. 11). Coelomoducts, on the other hand, are formed from the coelomesodermal tissue, after it has hollowed out to form a coelomic cavity. The cavity develops as a space in which to house the genital products, and a portion of its wall pushes outwards

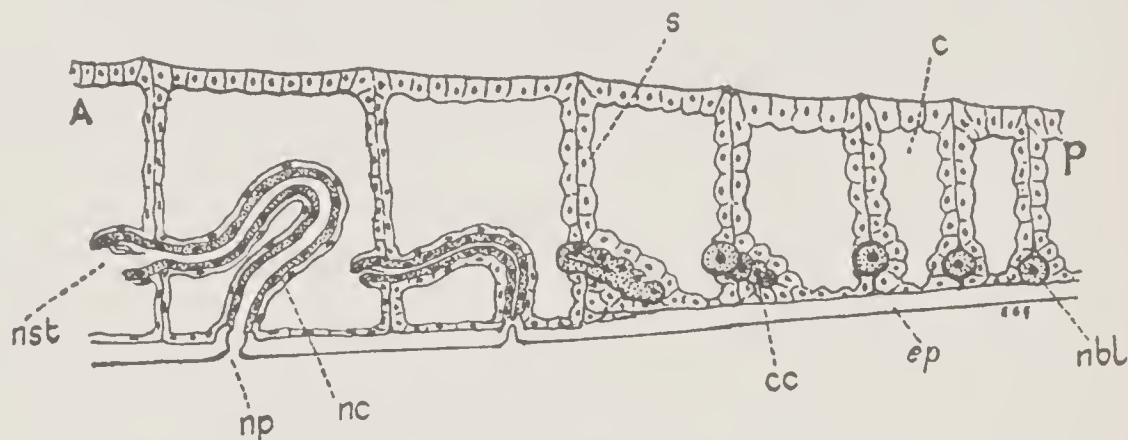


TEXT-FIG. 10. Diagrams of nephridia and coelomoducts in (a) Platyhelminth, (b) Nemertine, (c) Annelid.

bv. lateral blood-vessel; c. coelom; cd. coelomoduct; dv. dorsal longitudinal blood-vessel; flc. flame cell; g. gonad; gp. genital pore; int. intestine; ln. lateral nerve; lnc. longitudinal nephridial canal; mt. metanephridium; n. ventral longitudinal nerve; nc. ventral longitudinal nerve; np. nephridiopore; nst. nephridiostome; p. proboscis; pn. protonephridium; sol. solenocyte. (From Goodrich³⁴.)

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to open on the surface of the animal. Broadly speaking, though not with complete truth, nephridia are tubes which develop from without inwards and which, typically, end blindly; coelomoducts are evaginations from the wall of the coelomic cavity. Nephridia are primarily excretory in function; coelomoducts are primarily for the purpose of conveying the genital products to the exterior, though secondarily they



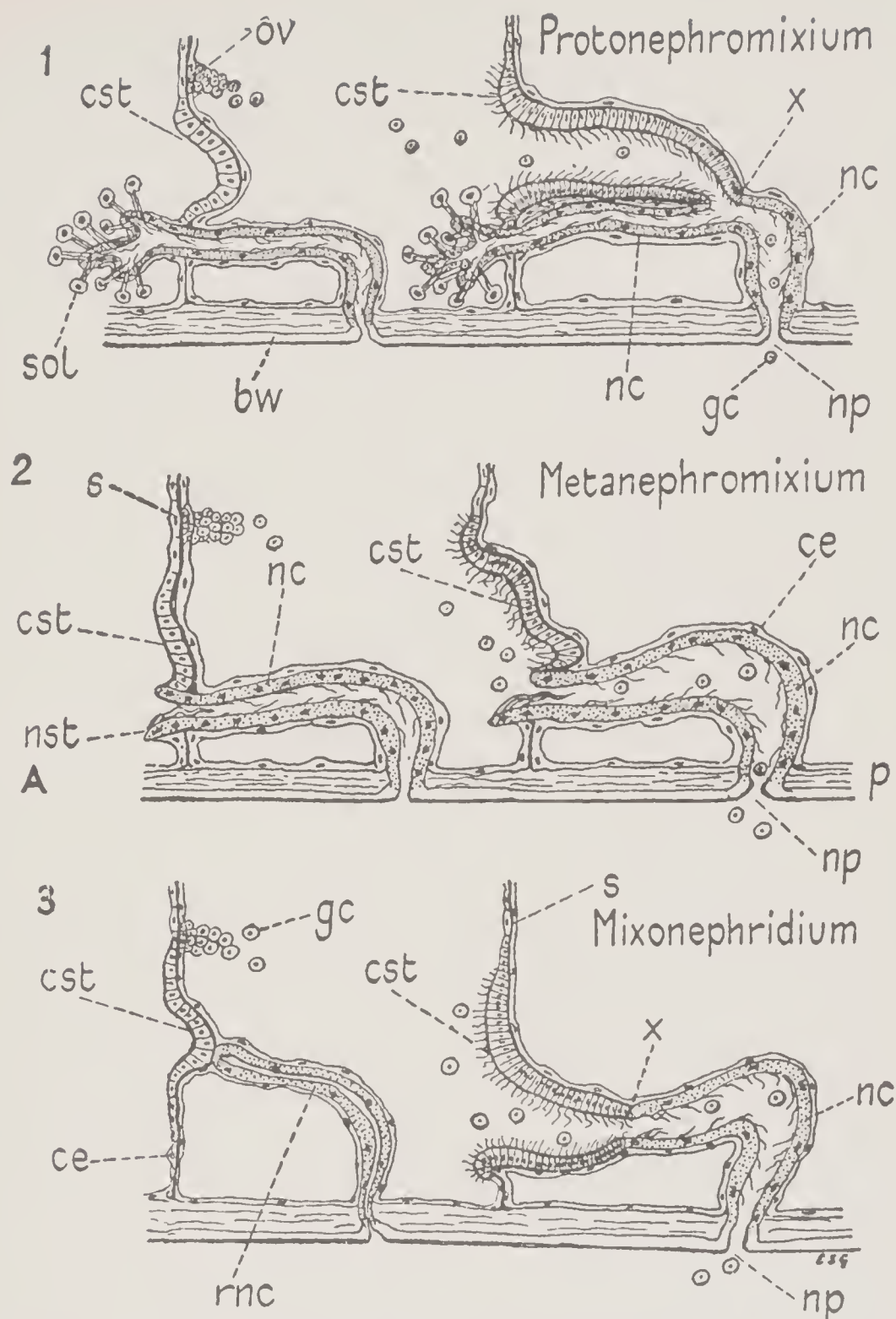
TEXT-FIG. 11. Diagram of development of metanephridium in *Oligochaeta*.

A. anterior; P. posterior; c. coelom; cc. canal cells; nbl. nephridioblast; nc. nephridial canal covered with coelomic epithelium; np. nephridiopore; nst. nephridiostome; s. septum. (From Goodrich³⁴.)

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may perform excretory functions. This description, however, greatly over-simplifies the picture. Both nephridia and coelomoducts occur in worms, molluscs, and many other groups, and there are many variations on the original theme. Nephridia may not always end blindly, but may open into the coelomic cavity by means of a ciliated nephridiostome (see Text-fig. 10), which is often very similar in appearance to the typical ciliated coelomostome which forms where the coelomoduct leaves the coelomic cavity. In some species fusion may take place between the two tubes, and this culminates eventually in a mixonephridium, as shown in Text-fig. 12. In *Peripatus* there are segmental coelomoducts (the coxal glands of Gaskell) but no nephridia. In *Amphioxus*, on the other hand, nephridia preponderate and actual coelomoducts, as tubes opening to the exterior, are reduced to a minimum; in fact the openings through which the genital products escape into the atrium may be specially developed for the purpose and not strictly homologous with coelomoducts in the usual sense.

Both systems of tubules are fundamentally segmental. Coelomoducts in lower animals tend to remain simple, while nephridia may show complex branching. Bahl^{3,4,5} has described in certain earthworms nephridia which do not open on to the outer surface of the body but which open into the alimentary canal. This he considers to be an adaptation related to the conservation of water by the animal. He has also described how in some worms the segmental nephridium may split up and form numerous separate meronephridia with secondary openings

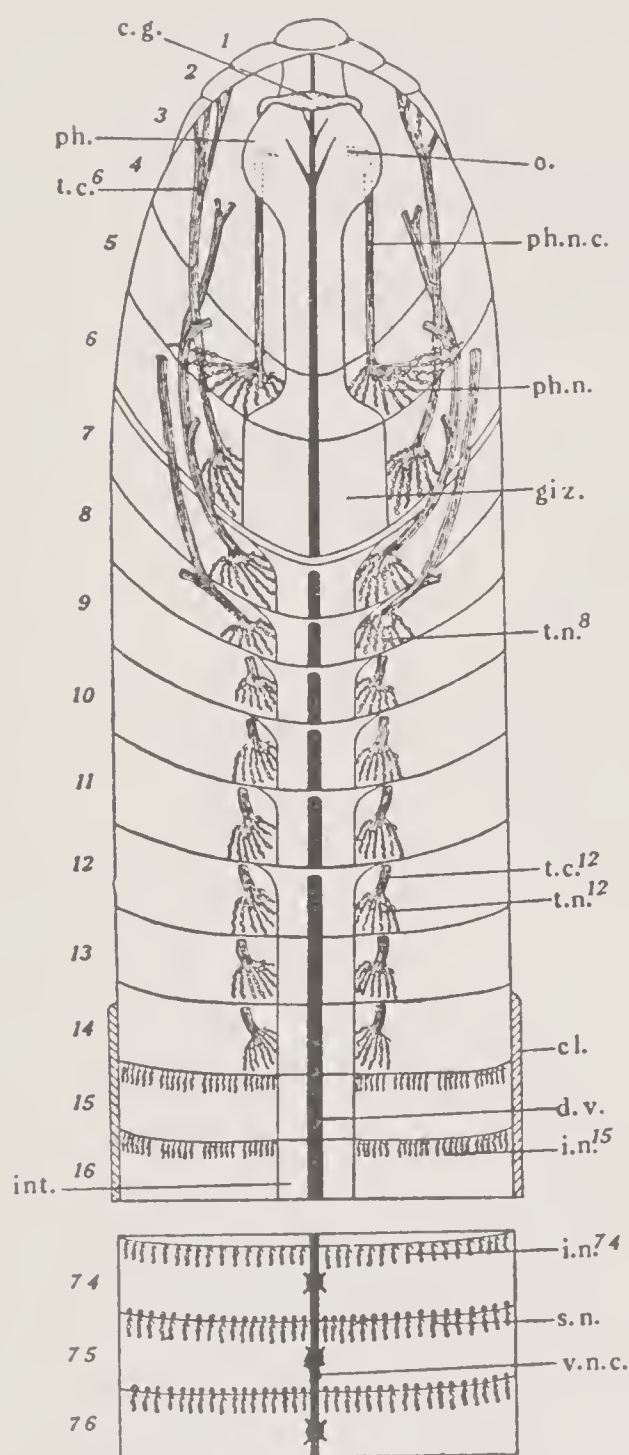


TEXT-FIG. 12. Diagrams showing structure and formation of nephromixia by combination of coelomoduct with nephridium.

A. anterior, young stage before combination; p. posterior, combination completed; bw. body wall; ce. coelomic epithelium; cst. coelomoduct; gc. germ cell; nc. nephridial canal; np. nephridiopore; nst. nephridiostome; ov. ovary; rnc. rudiment of nephridium; s. intersegmental septum; sol. solenocyte; x. point where coelomoduct opens into nephridial canal. (From Goodrich³⁴.)

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to the surface. Such properties of primitive nephridia may be important in considering what happens to the nephridial system in the evolution of



TEXT-FIG. 13. Simplified plan of nephridial system in *Megasclex cochinensis*.

c.g. cerebral ganglia; cl. clitellum; d.v. dorsal vessel; giz. gizzard; i.n. integumentary meronephridia; int. intestine; o. concealed opening of pharyngeal duct into lumen of pharynx; ph. pharynx; ph.n. pharyngeal meronephridia; ph.n.c. ductules of pharyngeal meronephridia; s.n. septal meronephridia; t.c. ductules of exonephric tufted meronephridia; v.n.c. ventral nerve cord. (From Goodrich³⁴, after Bahl⁵.)

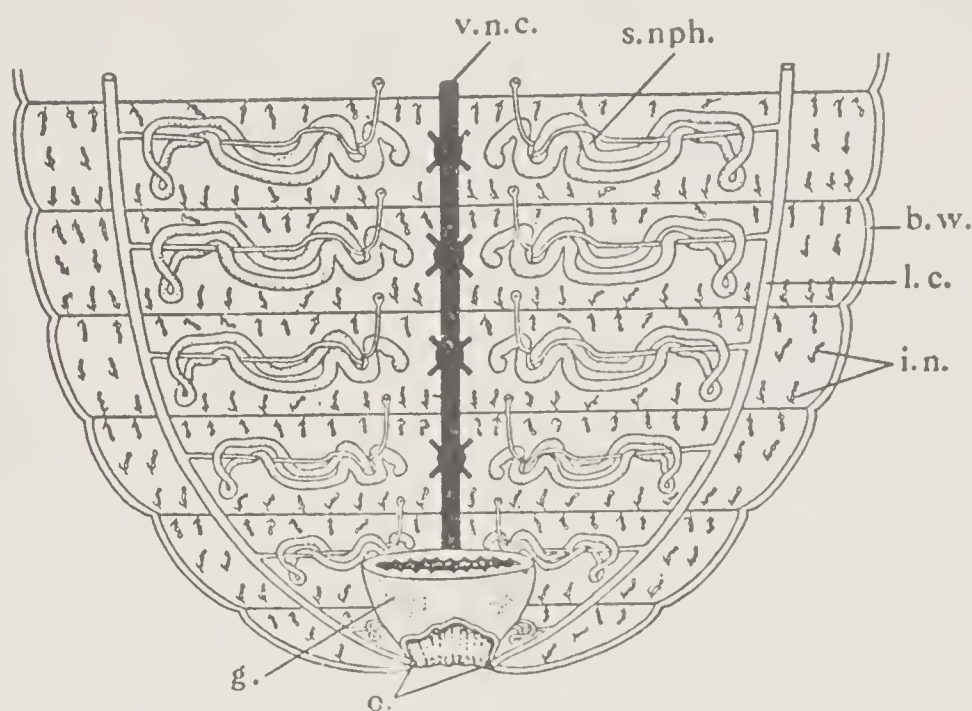
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vertebrates (Text-fig. 13). Another variation on the simple plan is seen when coelomoducts lengthen and travel through posterior segments before opening to the exterior. In this process they often receive contributions from the cavities through which they pass on the way. Similar common ducts may occur by fusion of the segmental nephridial tubes, and the final opening of the tube may be into the alimentary canal or into a cloaca, a point of some possible significance in considering the homology of the archinephric duct in primitive vertebrates. This arrangement is illustrated in *Hoplochaetella bifoveata* (Text-fig. 14).

Nephridia and coelomoducts, though differing widely in certain directions, have much of their general behaviour in common, and it is not always easy to decide whether a given tube is a nephridium, a coelomoduct, or a mixonephridium, unless its embryological origin can be determined. Text-fig. 12 shows the manner in which the two tubes may arise, and at first sight the development appears very different. When, however, the fundamental nature of the cells is considered, the difference may be found to be not so great, and it is worth while at this point to examine a little more closely what is meant by the differentiation of cells into their embryological layers and groups, and how the embryological origin relates to the physiological properties of the cells.

Observations on tissue cultures from the organs of vertebrates emphasizes that cells may be divided into certain definite groups according

to their manner of growth, their mode of locomotion, their nutritional requirements, their reaction to one another, and their response to changes in their environment. Moreover, these groups of cells are not the same as those into which the tissues are subdivided by the usual division into germ layers. The main groups of cells according to their properties in culture are epitheliocytes, and it is probable here that



TEXT-FIG. 14. Semi-diagrammatic drawing of nephridial system in last six segments of *Hoplochaetella bifoveata*. Dorsal view.

b.w. body wall cut open and spread out; *g.* hind-end of gut almost entirely removed; *i.n.* small integumentary meronephridia; *l.c.* longitudinal lateral excretory canal opening to exterior at *o*; *s.nph.* large open septal nephridium opening into lateral canal; *v.n.c.* ventral nerve cord. (From Goodrich³⁴, after Bahl⁴.)

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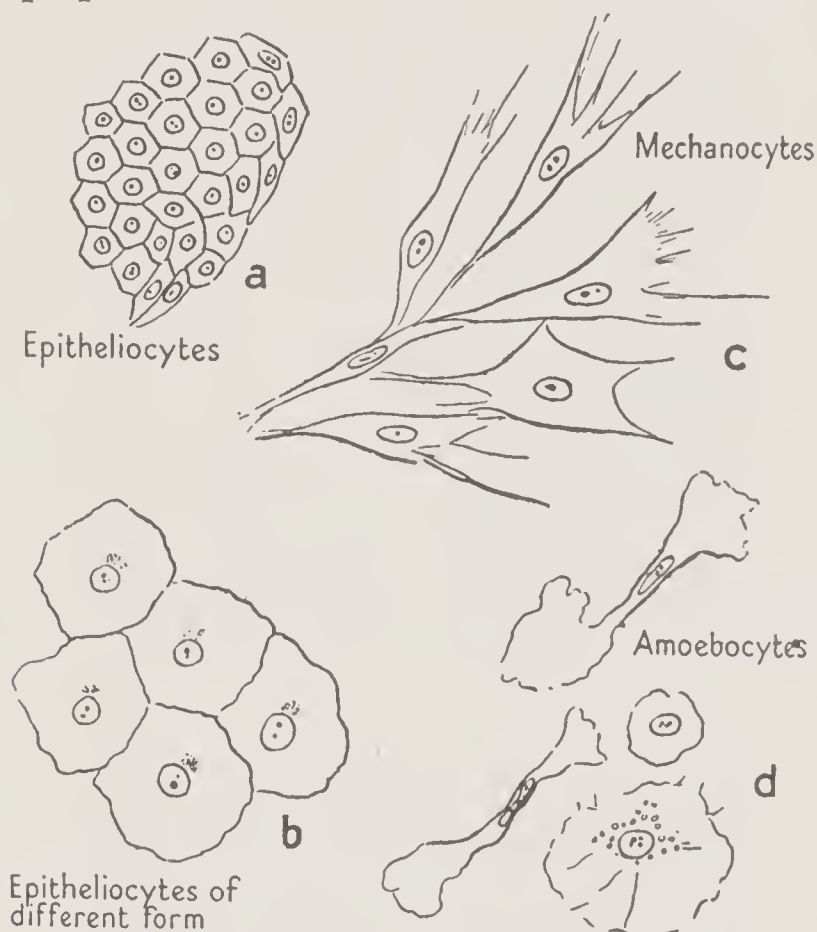
there may be two types (as exemplified by the growth of skin or intestine on the one hand and endothelium of blood-vessels on the other), mechanocytes (fibroblasts), which arise from the connective tissues, and amoebocytes, arising from the monocytes of the blood or the wandering cells of connective tissues. Some of the salient properties and characteristics of these groups are set out in Table I, and it is clear that the differences between them are fairly deep-seated. Moreover, it is important to realize that typical epitheliocytes may be obtained from either ectoderm (e.g. skin), endoderm (e.g. intestine), or mesoderm, for the kidney behaves *in vitro* as a typical epithelium. On the other hand, mechanocytes and amoebocytes are both confined to the mesoderm, and more nearly correspond to the cells of the mesenchyme. Text-fig. 15 illustrates the salient morphological characters of these groups of cells. The groups may be roughly described as comprising the surface elements, the mechanical and structural elements, and the wandering cells, and if the structure of such simple organisms as the calcareous sponges is examined it will be seen that the cells of these organisms show the same

TABLE I

<i>Epitheliocytes</i>	<i>Amoebocytes</i>	<i>Mechanocytes</i>
Cells adherent	Cells separate	Cells form network
Colonies unite	Colonies remain separate	Colonies unite
Cells move by gliding of the whole sheet	Cells move by 'random' amoeboid movement	Cells glide separately
Multiply in serum	Multiply in serum	Only survive in serum
—	Fresh serum causes cell divisions; latent period 20 hours	Embryo tissue juice required to cause cell division; latent period 10 hours
—	Survive in 1/300,000 arsenious oxide	Killed by 1/300,000 arsenious oxide
Not inhibited by malt extract	—	Inhibited by malt extract
Non-phagocytic, except certain groups	Phagocytic	Non-phagocytic
Keratin as protein framework of the cell	—	Collagen produced as fibres in external medium
Mucin produced within the cells of certain groups	—	Mucin produced as inter-cellular matrix
Mucin contains glucosamine	—	Mucin contains galactosamine
Carcinoma-producing	?	Sarcoma-producing

subdivision into epithelium (actually, primary and secondary epithelia), structural scleroblasts, and wandering amoebocytes (Text-fig. 16). The amoebocytes in the sponges include the germ cells and those cells which give rise to new individuals by gemmule formation. In the development of sponges the cells are at first nearly all adherent to one another as an epithelium of ciliated blastomeres, but in the posterior part of the blastula certain cells, which may still form an epithelium but do not possess or have lost their cilia, are somewhat larger and are classed as archaeocytes. In *Clathrina blanca* there are but two of these cells at the posterior end (Text-fig. 17a), but in other species they may constitute most of the posterior half of the blastula. Sooner or later these cells tend to leave the surface and to drop into the interior, where they become recognizable as amoebocytes, gemmule cells, or germ cells. The ciliated cells of the anterior end show, at a later stage, a tendency to lose their cilia and to drop inside the blastocoel, and it is from these cells that the scleroblasts and supporting cells of the body are derived, i.e. the mechanocytes. These potential mechanocytes may, as in the *Leucosoleniidae*, also be concentrated in the posterior region of the embryo, taking the place of the amoebocytes, which in these species have already sunk into the blastula cavity at an earlier stage.

These observations suggest that in such an organism as *Clathrina blanca* there has been a very early differentiation into two distinct groups of epithelial cells—the blastomeres of the anterior end, which yield mechanocytes, and those of the posterior end, which yield amoebocytes. Their properties may be, in part, determined by their position

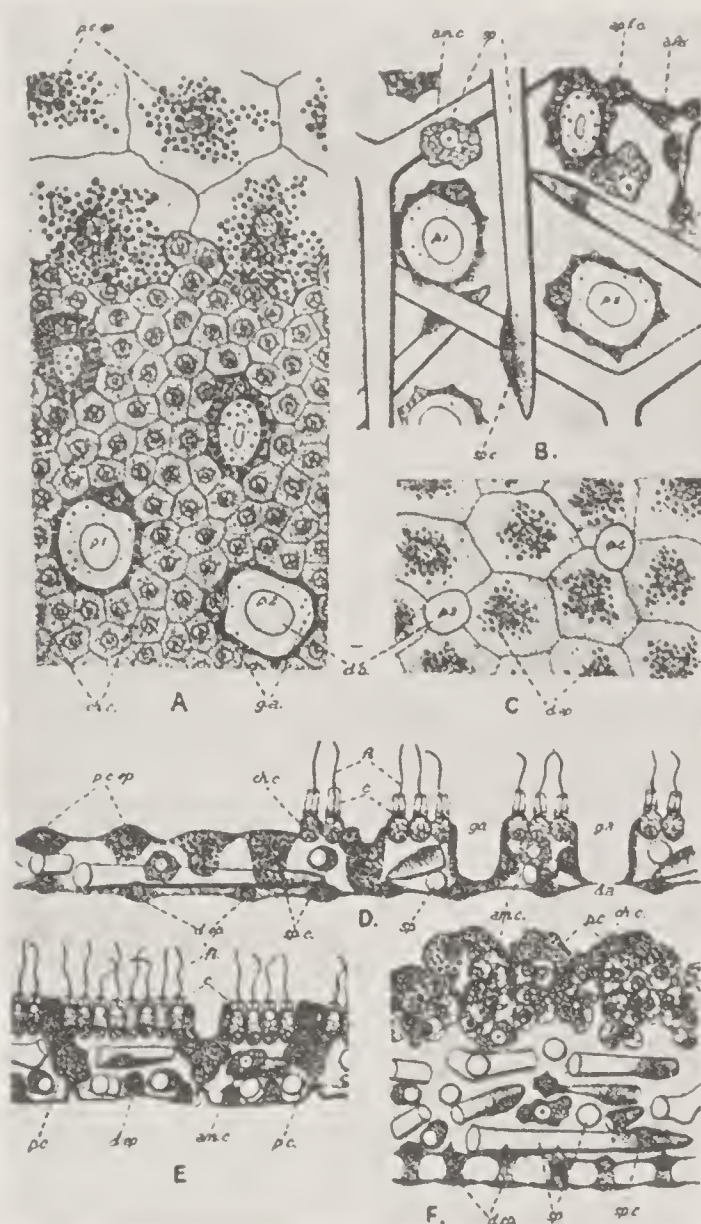


TEXT-FIG. 15. The primary groups of cells as seen in tissue cultures.

a, epitheliocytes; b, epitheliocytes, probably secondarily derived from mechanocytes; c, mechanocytes; d, amoebocytes.

in the embryo, but this need not necessarily be so. This early differentiation may be paralleled in the flagellate protozoa by the change of form between ciliated individuals and amoeboid individuals which occurs in such organisms as *Mastigella* and the *Rhizomastigina*, and seems to be related to reproduction and the formation of gametes (Text-fig. 18). On the other hand, the suggestion of Baker⁹, that the early metazoa would require to produce a change from a more or less saprophytic existence to a more aggressive type which would need the development of cells designed for nutrition and excretion, may be relevant to the origin of the cells which give rise to the amoebocytes. The two types of blastomeres of the sponge embryos may be conveniently classed as type A and type B, of which the former are mechanocyte producers and the latter are those from which amoebocytes arise. Although it is unlikely that the *Porifera* are on the direct line of metazoan development, this subdivision of their cells into types A and B and derivatives from these types is probably one of cardinal importance; and something

very similar has occurred in the evolution of the metazoa. It is interesting that, in cultures of tissues from vertebrates, mechanocytes depend for their growth upon extracts of other tissues or upon the proximity of amoebocytes, whereas amoebocytes live on simpler media and require

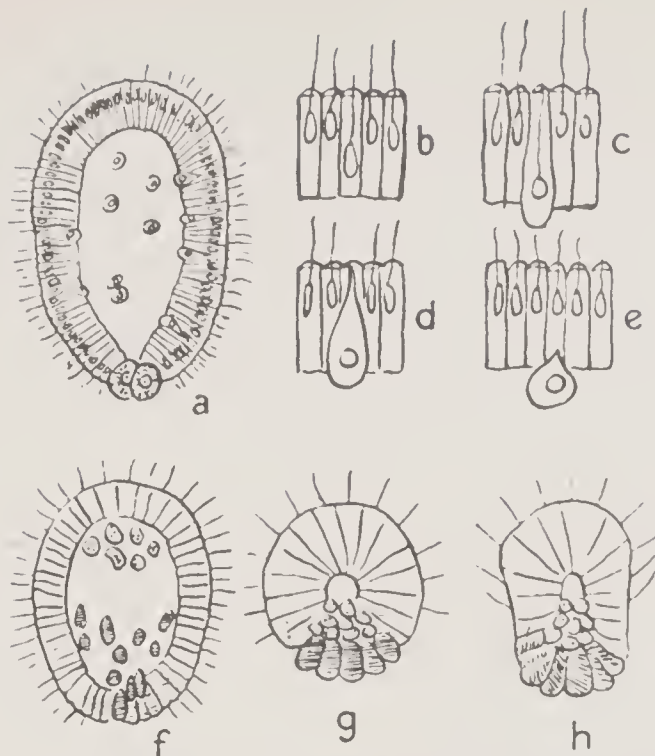


TEXT-FIG. 16. Histology of the body wall of a calcareous sponge (*Clathrina coriacea*).

A. body wall seen from the inside in the region of the oscular rim; B. portion of A, showing the same three pores, but with the collar cells removed to show the underlying parenchyma; C. same portion of the body wall seen from the outside, to show the flat epithelium; D. longitudinal section of the body wall in the region of the oscular rim, fully expanded; E. section of the body wall, slightly contracted; and F. very contracted. am.c. amoebocytes; ap.f.c. apical formative cell; b.f.c. basal formative cell; c. collars of (ch.c.) choanocytes; d.a. dermal aperture of pore; d.ep. dermal epithelium; fl. flagella; g.a. gastral aperture of pore; p¹. and p². pores; pc. porocytes; p.c.ep. porocytic epithelium; sp. spicule; sp.c. spicule cell or scleroblast. (From Michin⁵⁰.)

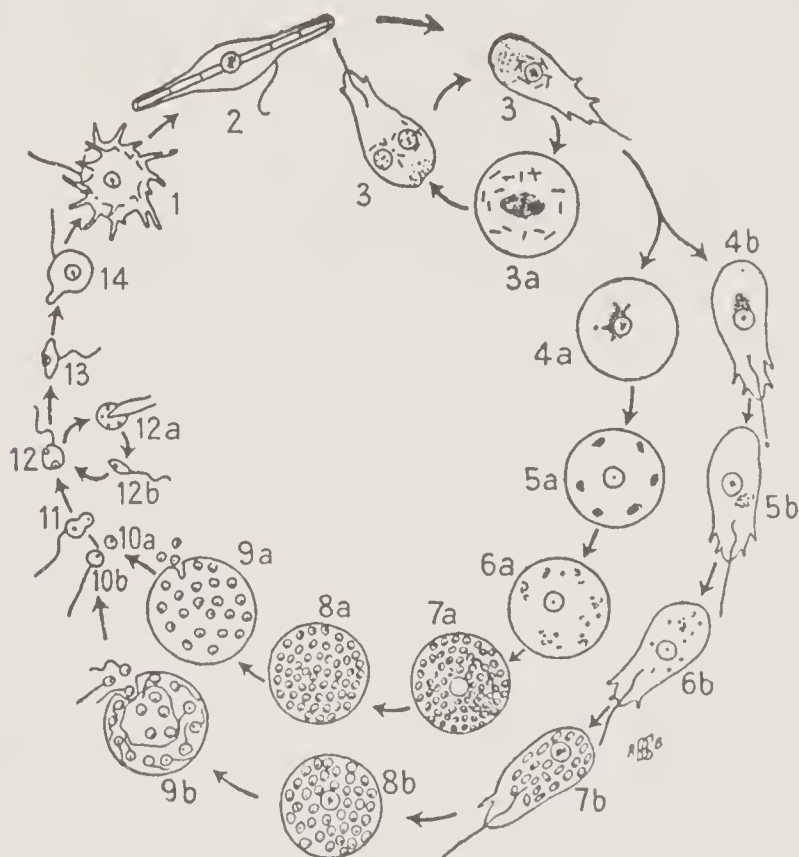
serum only. Needless to say, the stage at which this differentiation into two groups of blastomeres occurs varies widely in different groups of animals. In echinoderms all the blastomeres are equi- and toti-potential for a very long time.

Sometimes, as in the sponges, there is a clear morphological and spatial separation between the two types of epithelial cells; but this



TEXT-FIG. 17. Cell-types in the larvae of sponges (after Minchin⁵⁰).

a. *Clathrina blanca*. Two archaeocytes are seen at the posterior end, and mechanocytes are seen leaving the surface epithelium; b. c. d. and e. stages in the formation of a mechanocyte; f. invagination of mechanocytes and amoebocytes in *Clathrina reticulum*; g. and h. two stages in the development of *Leucosolenia*. In f, g, and h the shaded cells are potential mechanocytes; the dotted cells are amoebocytes which have already sunk into the blastocoel cavity.



TEXT-FIG. 18. Life-cycle of *Mastigella vitrea*.

1, 2, and 3, different forms assumed by the adult 'vegetative' type of individual; 3a and 3b, reproduction by binary fission; 4-10, gamete formation; a, microgamete formation, and b, macrogamete formation; 11, fusion of the gametes; 12, 12a, and 12b, multiplication by binary fission of the monad-like zygote; 13 and 14, growth of the monad-like form, after a period of multiplication, into the adult mastigamoeba form. (From Minchin⁵¹, after Goldschmidt.)

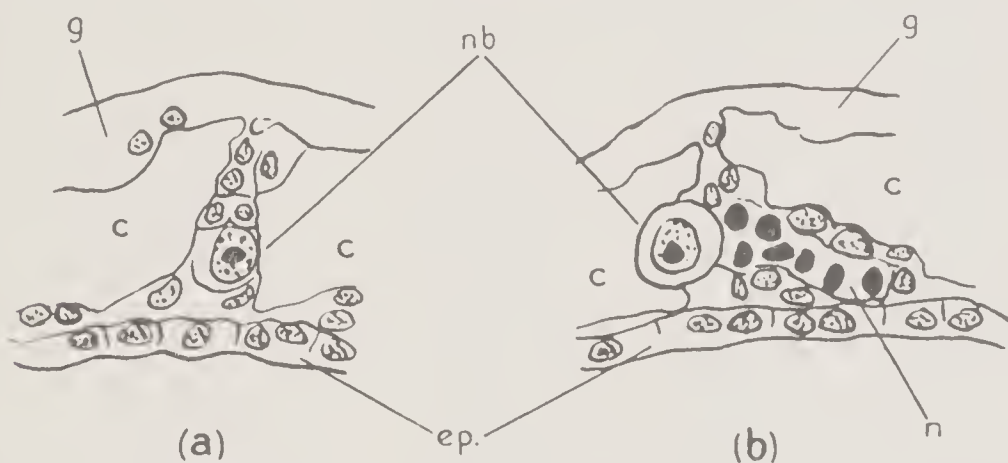
may not always be the case, and there are many reasons for believing that the superficial epithelium, both ectodermal and endodermal, of the adult metazoan organism may continue to contain derivatives from both types of cell in differing proportions. In this connexion it is worth noting the very common occurrence of pairs of cell-types in the surface membranes of the body, e.g. serous and mucous cells, ciliated and non-ciliated (often goblet) cells, sensory and supporting cells, rods and cones, acidophil and basophil cells, duct cells and alveolar cells, α and β cells, &c., for these may again be the result of the primary dichotomy seen in the superficial epithelium of the embryos of primitive animals such as the sponges: in other words, they may be derivatives of types A and B blastomeres. How, then, do these ideas on the constitution of the surface epithelium affect the meaning of the term mesoderm or, more strictly, the mesenchyme? The answer to this question clearly affects the manner in which one regards the embryological origin of such things as coelomoducts and nephridia.

The mesoderm and mesenchyme of different animals may arise in a variety of ways, e.g. by invagination, delamination, or by the dehiscence of individual cells. The term mesoderm is best used to describe a definite layer of cells with epithelial characteristics comparable with ectoderm and endoderm. Mesenchyme is then applied to those cells which detach themselves from any of the three layers, and form an inner mass of cellular tissue, generally without epithelial arrangement. Both mesoderm and mesenchyme may be expected to contain cells derived from the two main epithelial groups, i.e. potential or actual mechanocytes, and potential or actual amoebocytes. When, as in *Amphioxus*, much of the mesoderm separates by out-pocketing from the archenteron it is essentially an epithelial tissue, and blastomeres of type A and type B are probably both present in the walls of the coelomic sacs. The head mesenchyme and ectomesoblast, on the other hand, derived by direct dehiscence from ectoderm, may be largely derived from blastomeres of type A, as this type tends to be concentrated in the anterior end of the animal, and may therefore consist very largely of cells of the mechanocyte type, while the coelomesoblast derived from the mesodermal epithelium would be likely to contain far more of the amoebocyte type of cell.

It seems, therefore, that the properties of mesoderm and mesenchyme may be determined not only by the manner of their formation but also by their cellular content. In the case of the nephridia these tubes are described as being formed of ectomesoblast, and though they may therefore contain cells which are mostly of the mechanocyte type (from blastomeres type A), there may be amoebocytic cells there as well (from blastomeres type B). A special cell, the nephridial cell, is generally clearly recognizable among the other cells of the nephridial rudiment as

a cell of different type (Text-fig. 19), and a large part of the nephridium, including the flame cells, or solenocytes, and the nephridiostome, when such is present, seem to be formed from this cell.

Coelomostomes and coelomoducts are derived essentially from the coelomic epithelium and coelomesoblast and are thus in this respect quite different from nephridia, but they may, in fact, be much more similar than they appear. They are formed by outgrowth and tubule formation from blastomeres which have been carried inwards in the



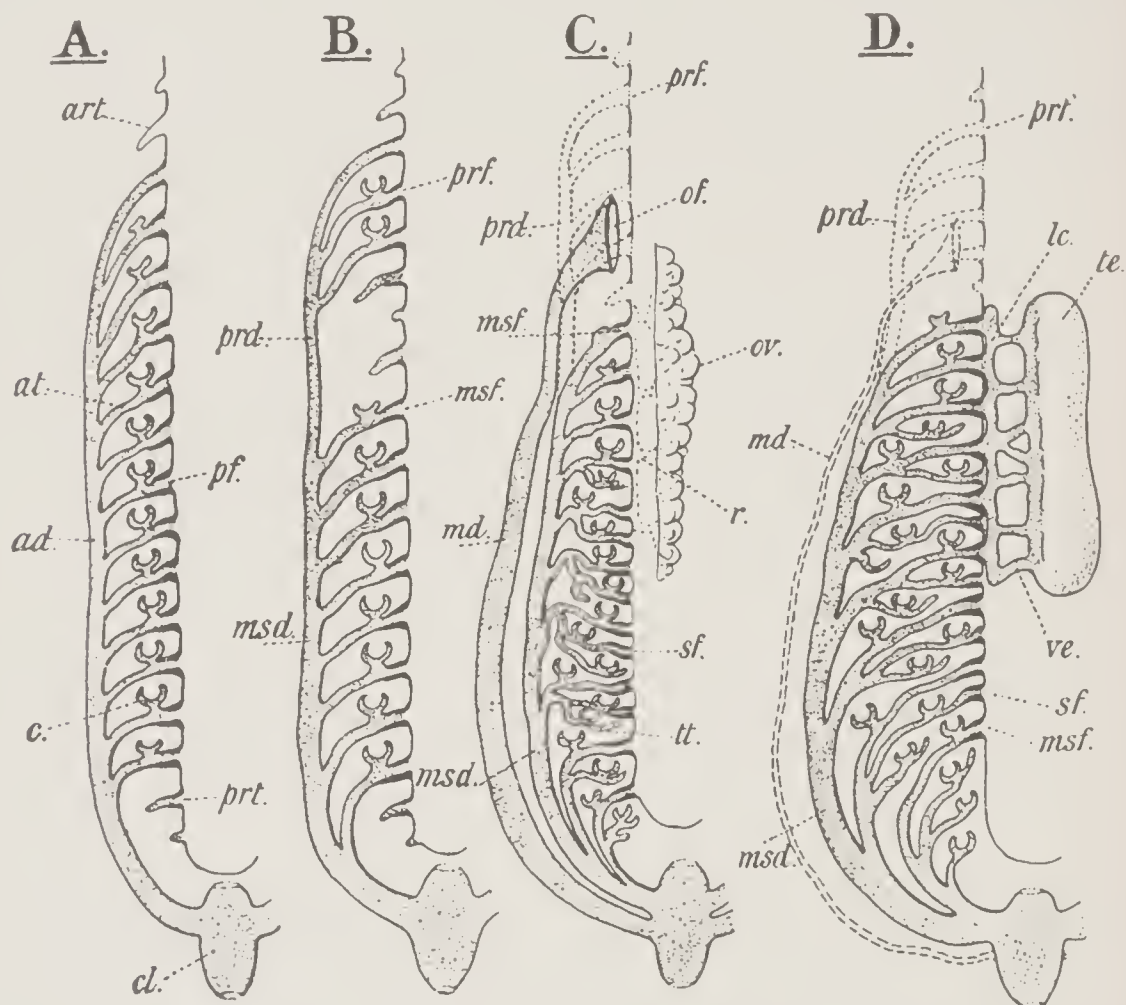
TEXT-FIG. 19. Longitudinal sections of septa in *Tubifex rivulorum*, showing the relation of nephridioblast to the cells of the septum.

(a). septum at young stage; (b). septum at older stage; c. coelomic cavities; ep. epithelium; g. gut wall; nb. nephridioblast which will survive as the funnel cell; n. other nephridial cells. (Re-drawn after Goodrich³⁴.)

formation of the wall of the coelomic cavity. This wall may clearly contain cells belonging to both types A and B, and thus the coelomoduct rudiment and the nephridium rudiment are, from this point of view, closely similar, and much of the distinction based on the different spatial origin of the two disappears. The physiological characteristics of the basic cell types may be just as important as the secondary characteristics forced on the cells by virtue of their anatomical position in the animal; at least, there is bound to be a balance between these two major determining factors.

With these considerations in mind, it is now appropriate to turn to the structure of the urinogenital system of the primitive vertebrate. The essential structure is shown in Text-fig. 20. It consists of a series of tubes which are more or less segmentally arranged. This is particularly evident internally where the tubes are derived from the coelom, but the tubes join together to open posteriorly by a common duct, rather like the common nephridial duct of *Hoplochaetella*. The arrangement was presumably strictly segmental at one stage, as in Text-fig. 14, and each tube may even have had a separate exit to the exterior, but in the earliest known vertebrates, as represented by the ammocoete larva, the tubes are clearly divided into aggregates as a pronephros and a mesonephros opening by a common duct leading posteriorly to the

cloaca. They develop as coelomoducts, that is, by outgrowth from the coelomic cavity. The manner of their development, disregarding the fact that they do not remain as separate segmental structures, has an extraordinarily close parallel in the coelomoduct system of *Peripatus*, as



TEXT-FIG. 20. Diagrams of urinogenital system in *Craniata*.

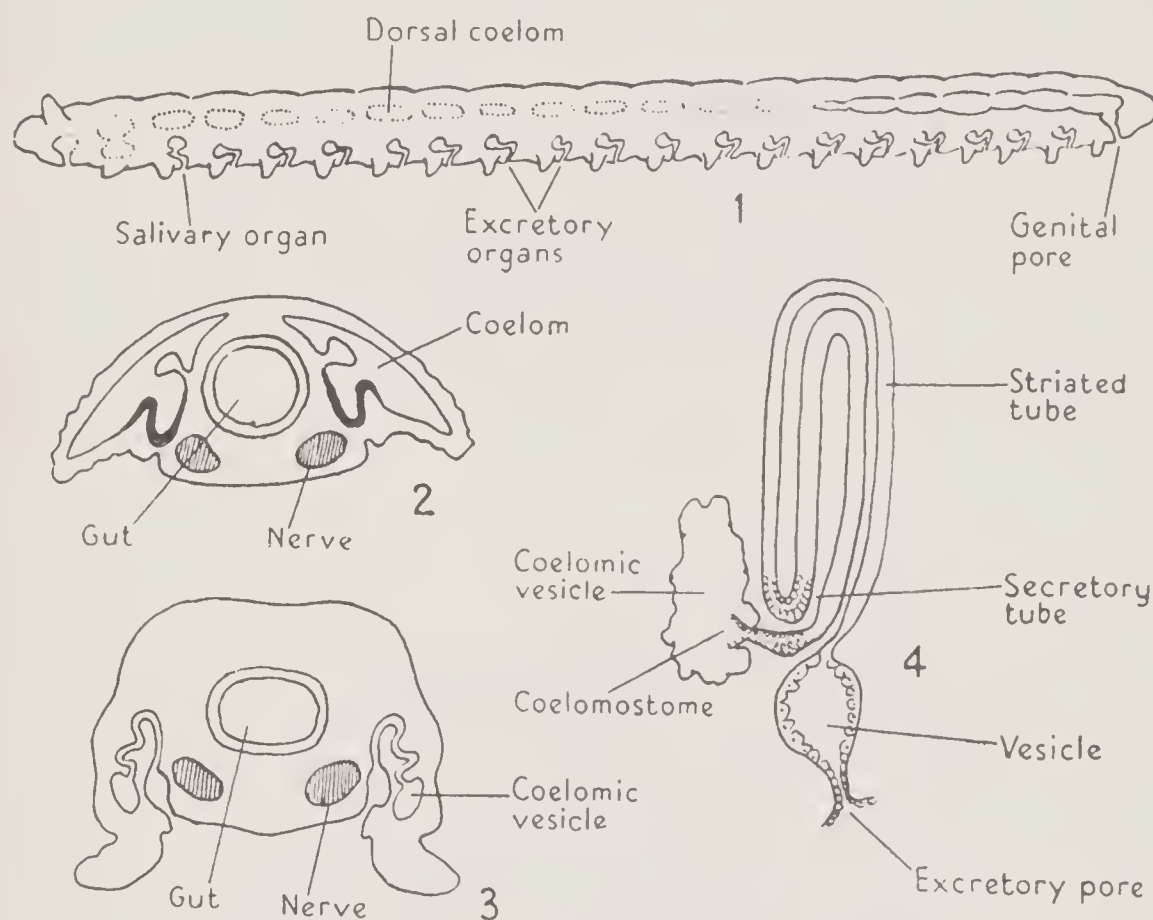
A. Hypothetical ancestral stage with continuous archinephros. B. Cyclostome with pro- and mesonephros. C. Female adult gnathostome. D. Male adult gnathostome. ad. archinephric duct; art. anterior vestigial tubule; c. Malpighian capsule; cl. cloaca; lc. longitudinal canal; md. Mullerian duct; msd. mesonephric duct; msf. mesonephric funnel; of. oviducal funnel; ov. ovary; pf. coelomostome or peritoneal funnel; prd. pronephric duct; prf. pronephric funnel; prt. posterior vestigial tubule; r. vestigial vasa efferentia; sf. secondary funnel; te. testis; tt. tertiary tubule; vs. vas efferens. (From Goodrich³⁴.)

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was pointed out by Gaskell (Text-fig. 21). While outgrowths from the coelom clearly enter into their composition and have led to the belief that the tubes are essentially coelomoducts, there are reasons for believing that the tubes may be more in the nature of mixonephridia, because they show some of the properties which, in lower animals, are more characteristic of nephridia. The capacity for secondary branching is one such character.

At this point it is essential to emphasize that not only are there cells of different intrinsic morphological and physiological types, but there are also different sorts of behaviour, which may be inherited by cells or which may be imposed upon them by virtue of their position and

surroundings. All these factors determine the formation of a particular structure or organ. Cells in different regions clearly show different patterns of behaviour, as, for example, a tendency to form tubes in a particular direction or to dehisce at a given time. Some of these patterns are inherent in the cells themselves, at any rate after a certain time; others are impressed upon them by local conditions.

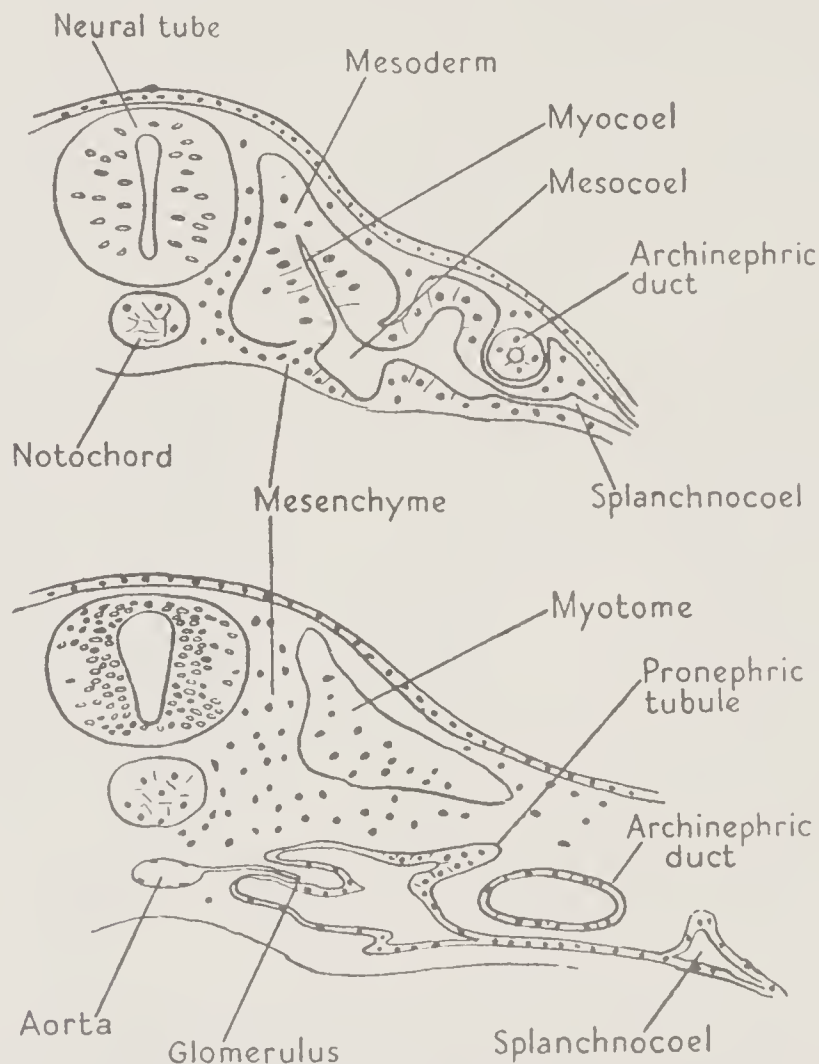


TEXT-FIG. 21. The coelomoduct system of *Peripatus*.

1. Diagram of *Peripatus* showing the position of the coxal glands. 2. Transverse section of the developing animal, showing the formation of the coelom and the development of the excretory organs. 3. Transverse section of the adult animal, showing the excretory organs. 4. The structure of the excretory organ. (Diagram taken partly from Goodrich²⁴, and partly from Gaskell³¹.)

It is worth while, therefore, to consider the formation of the pronephric and mesonephric rudiments in some detail and to study the relationships which these structures bear to the different parts of the coelom and to the mesenchyme and ectomesoblast. Text-fig. 22 shows the essential features in the development of the coelom and its derivatives in the region in which the pronephros develops in what may be regarded as a typical vertebrate. The coelomic cavity is divided into three main portions—the dorsal myocoel, the middle mesocoel, and the ventral splanchnocoel. The pronephros develops partly as a series of outgrowths of the mesocoel (the coelomic funnels) and partly from a rudiment of cells which may arise independently in the ectomesoblast (mesenchyme), and which forms the archinephric duct. The coelomic funnels fuse with this duct before it grows backwards to open at the

cloaca. There is no evidence that the archinephric duct is ever in direct contact with the ectoderm or is budded off from this layer as primitive nephridia may be, but there is good evidence that it may arise by condensation of the ectomesoblast or mesenchyme rather than by evagination from the coelomesoderm, so that the possibility that it represents

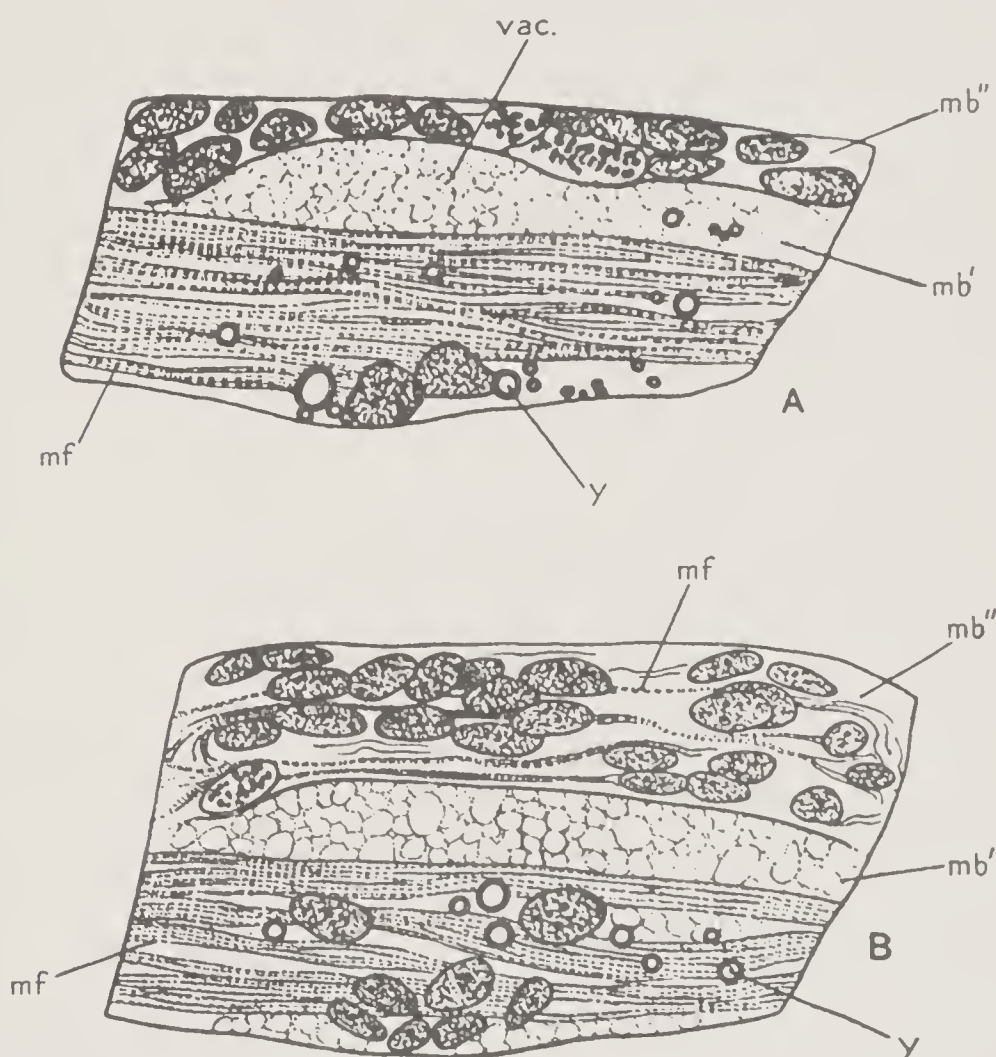


TEXT-FIG. 22. Diagram illustrating the development of the coelomic structures in the region of the pronephros in fish.

the remnants of an earlier nephridial system is not entirely ruled out of court.

The coelomic cavity, though it arises in higher animals by a split in a mass of mesodermal cells which have, earlier, separated from the endoderm, is fundamentally a part of the outside world. It was originally a part of the archenteron which became nipped off. In its primitive form, as in *Amphioxus*, it is surrounded by a special part of the endoderm, now given the name mesoderm, so it is worth considering the types of cells which it may be expected to contain. Primitively the cells are epithelial in character, and the coelomic cavity is surrounded by cells of epithelial type. These, later, tend to leave the epithelium proper and to sink into the underlying tissues, where they may give rise to muscles of various sorts or to the other organs and tissues usually described as mesodermal in origin. Cells of type A (see p. 461) seem

to be specially concentrated in the dorsal region of the coelomic wall where they give rise to the myotomes and so to the skeletal muscles. *In vitro*, these mostly grow as mechanocytes. In the ventral portion of the coelom also the type A cells presumably contribute to the formation of plain muscle. It is, however, worth while to consider whether the property of contractility and muscle formation is necessarily a



TEXT-FIG. 23. Differentiation of the myotome as seen in horizontal sections of *Lepidosiren* larvae.

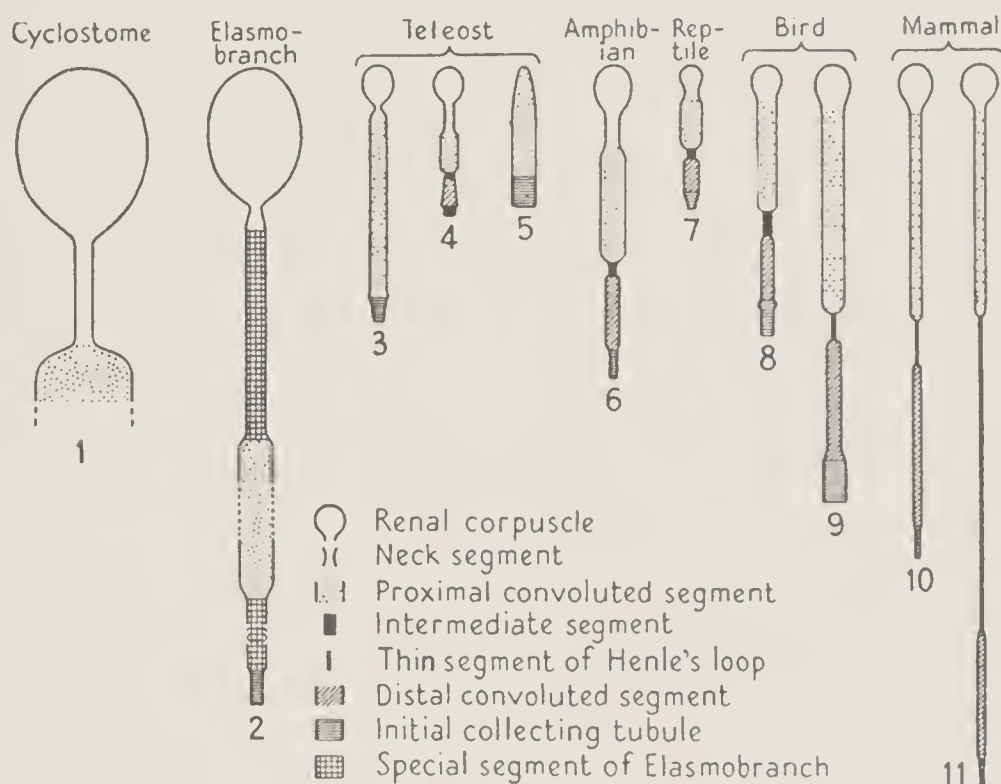
B represents a later stage than *A*. *mb'*, myoblast of inner wall; *mb''*, myoblast of outer wall; *mf*, contractile fibrils; *vac.*, vacuoles; *y*, yolk. (From Kerr⁴³.)

prerogative of type A blastomeres only. Some of the unaccountable appearances of amoebocytes in cultures of muscle and some of the differential responses of plain muscle to acetylcholine and adrenalin would become easier to understand if there were two types of cell involved in muscle formation, particularly of plain muscle, one perhaps derived from type A blastomeres and the other from type B. The development of muscle from the myotomes of *Lepidosiren* lends some support to this view (Text-fig. 23). In the mesomeric or nephrotome portion of the coelom it may be that both types of cells are present, as is suggested by the morphology of the cells to be the case in the nephridial rudiment in worms, but probably type B cells will be found

to preponderate. The germ cells, which are clearly in this category (type B), become concentrated in the middle region, and particularly in the dorsal wall of the splanchnocoel. It may be tentatively suggested, therefore, that the pronephric funnels are remnants of the coelomostomes (though it is possible that they are nephridiostomes) and that the cells which form them may belong to both types A and B, and that they will inherit a tendency to form evaginations from the coelomic epithelium. The archinephric duct, on the other hand, may represent the remains of the nephridial system and, again, may contain cells of both types; the duct will inherit a tendency to branch (see Text-fig. 10) and for the branches to end blindly in or near the coelom, or to end in ciliated funnels opening into the coelomic cavity. Moreover, there will be a tendency for the two systems to unite, as the coelomic funnel and archinephric duct so clearly do whatever the origin of the systems, in the formation of both the pronephros and the mesonephros. Both coelomostomes and nephridia, in their most developed form, consist of a ciliated coelomic or proximal portion and a non-ciliated distal portion. End-to-end union, therefore, or union at some other point, between coelomic outgrowth and archinephric duct may clearly give rise to tubes with complicated architecture such as are found in the nephron of the kidney. In mammalian kidneys there are five fairly distinct regions of each nephron in which the cell structure and probably cell function are clearly distinguishable; and these segments, in varying proportions, turn up in practically all vertebrates. They consist of a glomerular segment, a proximal tubule, generally with a brush border to the inner margin of the cells, an intermediate segment, a distal tubule, and finally the collecting tubes (Marshall⁴⁸). Their persistent presence in vertebrates of different groups strongly indicates inheritance from a tube of complex origin as would arise by end-to-end union of coelomoduct and nephridium (Text-fig. 24). On the other hand, it is only fair to point out that considerable complexity of structure may occur in protonephridia (Pantin⁵³), where the origin is probably from a simple nephridial rudiment containing two visibly different types of cell.

The functions of the nephridia and coelomoducts in lower animals were, respectively, to regulate the composition of the body fluid and to allow the escape of the genital products. At first there was only tissue fluid, and there were no coelomic spaces filled with fluid (Text-fig. 10*a*). As the coelom developed, its fluid became more and more copious, and we find the nephridia pushing nearer to or even into the coelomic cavities and assisting to maintain the composition of the contained fluid (Text-fig. 10*c*), and, in some animals, the nephridial tubes have even usurped the genital function of the coelomoducts. This last has generally been brought about by the coelomoducts fusing on to the nephridial tubes with the formation of nephromixia or mixonephridia (Text-fig. 12).

In vertebrates, as compared with invertebrates, the vascular system becomes relatively much more important in the economy of the body, and the regulation of the composition of the blood becomes more necessary; blood-vessels then become related to the coelomostomes. At first, a knot of capillaries (glomus or glomerulus) projects into the coelom near the mouth of a coelomostome. Later, this forms in a special chamber of the coelom, the nephrocoel, which is almost closed off from the

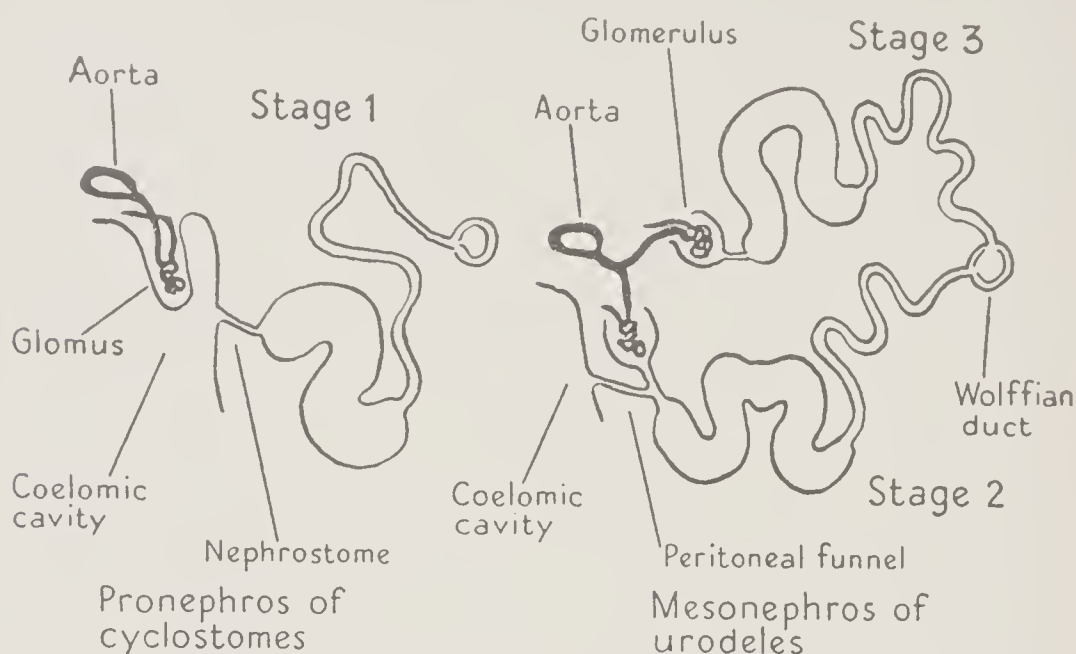


TEXT-FIG. 24. Schematic representation of nephron of different vertebrates. (From Marshall⁴⁸.)

main coelomic cavity. Finally, this separate chamber closes off entirely and the arrangement found in the higher vertebrates is developed. These stages are illustrated in Text-fig. 25. The first stage is found in the pronephros of cyclostomes, in amphibia, where the coelomostomes of the pronephros remain patent, and in a less perfect form in the pronephros of birds. The second stage is found in the mesonephros of urodeles. The possible mechanism of this development is illustrated in Text-fig. 26.

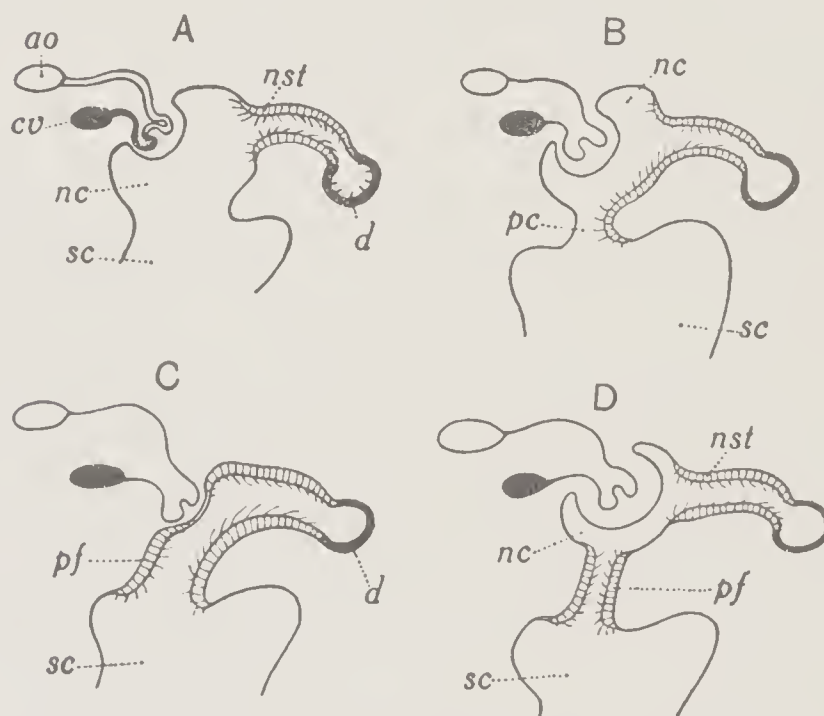
The perfecting of the third stage, as it occurs in mammals, with typical glomeruli filtering fluid into the closed Bowman's capsule, naturally means that the genital products have to find a new way of escape. In most higher animals the more anterior nephric rudiments degenerate as such, and the pronephros may never function. One coelomostome of the pronephros of the female, however, remains connected with the archinephric duct, and this forms the Mullerian duct and so provides an exit for the ovum (see Text-fig. 20). The duct subdivides into the proximal Fallopian tube which, be it noted in connexion with its origin

from the coelomic funnel, has a ciliated epithelium containing two distinct types of cell, and into the distal uterus whose epithelium shows



TEXT-FIG. 25. Stages in the development of the glomerulus and Bowman's capsule in relation to the coelomic cavity and the nephron.

(After Gérard and Cordier³².)



TEXT-FIG. 26. Diagrams of segmental excretory tubules (coelomoducts) of vertebrates.

A, usual condition of pronephric tubule; *B*, hypothetical intermediate condition; *C*, supposed primitive condition; *D*, complete tubule, pronephric or mesonephric. *ao*, dorsal aorta; *cv*, cardinal vein; *d*, longitudinal duct in transverse section; *nc*, nephrocoel; *nst*, nephrocoelostome; *pc*, communication between nephrocoel and splanchnocoel, *sc*., corresponding to the peritoneal funnel, *pf*. (From Goodrich³⁴.)

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a remarkable tendency to throw out tubular gland-like processes. Again, in the uterine epithelium there are indications of the presence of two types of cell. Does the uterus therefore correspond to the nephridial

portion of an original mixonephridium in which the ova were sometimes delayed in their passage to the exterior and where Nature therefore began to provide for their welfare by the secretion of a nutrient fluid? Even this mode of exit for the ova, by way of the oviduct, may be secondary and, as such, dependent on an earlier failure on the part of the mesonephric tubules to perform the function satisfactorily, as will become evident in connexion with the development of the gonads themselves.

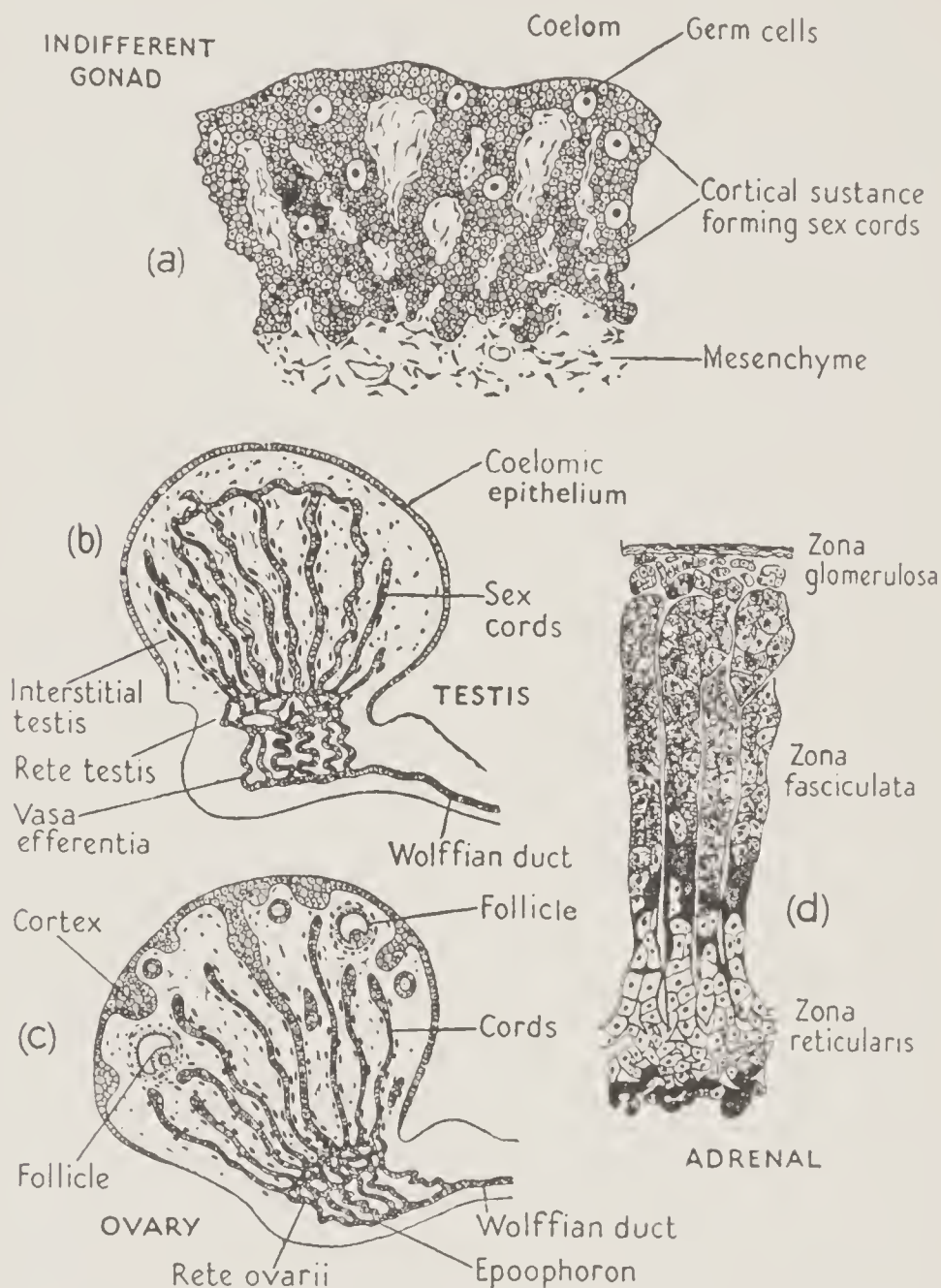
The series of segmental tubes of the next more posterior region gives rise to the mesonephros which in adult mammals is never functional as such, though it may be in birds. Its duct, however, persists, and the mesonephric rudiments become involved in the genital apparatus, so that, in the male, it is these tubes which provide a means of exit for the sperm. The mesonephros generally develops in much the same way as the pronephros, i.e. by coelomic outgrowths fusing with an earlier duct rudiment. Its further history will be considered later, with the development of the gonads.

The functional metanephric kidney, it should be noted, develops similarly still farther back. It often arises from nephrotome rudiments which are somewhat separate from the coelomic epithelium, and it appears to be an elaboration of the Wolffian duct system. It shows a great tendency to form increasing numbers of tubules by a process of branching, and in this way resembles the structures of nephridial origin.

In worms with well-developed coelomic cavities the germ cells are often found to be concentrated on the dorsal wall of the coelom. The coelom is, of course, the primary genital sac whose lining therefore contains the germ cells. These may reach the coelomic wall either singly or in mass from the endoderm, or perhaps from the mesoderm, or even from the ectoderm, but it is important to remember that the coelom is, by definition, the genital sac; sooner or later it must contain the germ cells even if, by some secondary alteration in the time-table of development, they are not present in the coelomic epithelium from the start. When the cells ripen they may become loose in the cavity, which in most higher animals has enlarged as the general body cavity, and they eventually pass to the genital funnels and so to the outside. How, then, is the gonad of the vertebrate related to this simple system?

Embryologically, the gonads develop in most higher vertebrates as genital ridges. These are thickenings of the dorsal wall of the splanchnocoel in the region near the nephrocoel and nephrotome rudiments. At first there is an increase in the cells of the underlying mesenchyme stroma, and the epithelium of the splanchnocoel becomes thickened. Cells then leave the epithelium and penetrate into the stroma, at first irregularly (Text-fig. 27*a*), but then they arrange themselves into definite cord-like structures. Eventually the structures diagrammatically

represented in Text-figs. 27 *b* and 27 *c* crystallize out from the masses of cells. A possible interpretation of this system seems to be that the germ cells become precociously enclosed in the coelomostomes and coelomoduct tubes, and that these sex cords, and perhaps rete testis and rete



TEXT-FIG. 27. Comparison between developing gonads and the adrenal cortex.

(a). Primordium of the gonad, showing the formation of sex cords and cortical substance.

(b). Developing testis.

(c). Developing ovary.

(d). Section through the adrenal cortex.

((a), (b), and (c) from Maximow and Bloom⁴⁹, after Kohn; (d), from Cowdry⁵⁰, after Goormaghtigh.)

ovarior, represent the rudiments of the coelomic epithelial outgrowths of these segments by which the germ cells are led to the archinephric duct; in other words, they represent the original coelomostomes and coelomoducts. In the male this system becomes patent in a few segments, and the sperm are led by way of the vasa efferentia and the mesonephric

or Wolffian duct to the exterior. In the female, however, perhaps owing to the increased size of the ovum, the germ cell does not use this pathway, but is secondarily shed back into the coelom, whence it is passed to the outside by the one specialized coelomostome remaining from the pronephros, which has become modified into the Fallopian tube. How much of the system of sex cords and rete is actually coelomic in origin and how much nephridial is difficult to determine. It is said (Torrey⁵⁶) that in grafts of the genital ridge a rete testis develops, while in grafts of the Wolffian body or mesonephric rudiment it does not. This might be taken to indicate that the rete testis is a derivative of the coelomic epithelial outgrowth rather than of the nephridial elements.

In the male gonad (Text-fig. 27*b*) the cells show a well-defined distribution and pattern of behaviour. First, there are those which form the rete testis, and these are initially arranged in columns but later form open tubes lined with ciliated epithelium. Secondly, there are those which form the sex cords. These later line the seminiferous tubes in which germ cell maturation occurs and in which the Sertoli cells are found. The actual tubules are formed of connective-tissue fibres, and these separate the contents of the tube from the general stroma, which consists of a few structural (mechanocytic) elements and also of the interstitial cells. The cords separate completely from the original coelomic epithelium and the mechanocytic cells of the stroma lay down a layer of fibres, the tunica albuginea, between the two. In this pattern it is important to consider the nature and origin of the cells in the various groups and their derivation from the coelomic epithelium in which type A and type B cells are probably present. This, however, is better deferred till the same problem can be attacked with reference to the ovary and the closely related adrenal gland.

In the ovary (Text-fig. 27*c*) the early development is, as already stated, almost identical with that of the testis, except that the cells continue to pass from the coelomic epithelium into the substance of the organ and no fibrous coat develops to separate the two. The rete ovarii and sex cords break up as such and the cells thus liberated form the interstitial cells of the ovary, while the later invaginated cells form vesicles and give rise to the ovarian follicles, in which they constitute the membrana granulosa.

The adrenal cortex develops in a very similar way. Adjacent to the genital ridge the coelomic epithelium forms the adrenal cortical rudiment. Coelomic epithelial cells leave the epithelium proper and sink down into the underlying stroma where they produce a pattern almost identical with that of the ovary and testis. Moreover, in the ovary the germ cells become recognizable as large round cells, generally surrounded by other cells from the coelomic epithelium (nurse cells), and in the adrenal rudiment it is not uncommon to find at one stage that there

are also large round cells located in much the same way (Uotila⁵⁸). These cells subsequently degenerate, and it is tempting to think that they may actually be germ cells which get into the adrenal cortical tissue because the adrenal cortical tissue is part of the same general tissue as the ovary and testis; it is, however, a part which has lost its primary function and has developed as an endocrine gland. In its later development the adrenal cortex forms a rete (zona reticularis), cell cords (zona fasciculata), and a follicular zone (zona glomerulosa) (Text-fig. 27 *d*).

In any discussion on the properties of the various cells in these organs the phylogenetic and ontogenetic approach emphasizes two main factors. In the first place, the cells must be derived from type A or type B blastomeres, and secondly, they may have been segregated into the coelomostome rudiment or into the nephridial rudiment and will have inherited a characteristic pattern of behaviour in each case. The problem is made more difficult because these two patterns are in many ways closely similar, for both coelomoduct and nephridium may achieve a ciliated coelomic funnel and a non-ciliated conducting tube, though they do so by different methods. There does, however, seem to be a close parallelism between the zona glomerulosa of the adrenal, the follicles of the ovary and perhaps Bowman's capsules, and the proximal convoluted tubules of the kidney. Bowman's capsules, it will be remembered, are nipped-off portions of the coelom. It should, however, be pointed out that the homology of the kidney structures is somewhat doubtful, since a great part of them is more likely to be nephridial in origin and have approached the coelom from the outside, rather than have arisen as outgrowths of its epithelium, so that Bowman's capsules may represent modified nephridiostomes. The close parallelism, on the other hand, which exists between the coelomoducts of *Peripatus* and the structure of the kidney in the early vertebrate argues against the view, and perhaps favours a coelomic origin for the proximal part of the kidney tubule; but the actual relationship of the coxal glands of *Peripatus* to the coelom of other animals is still a matter of dispute. The secondary multiplication of nephric tubules which goes on in the developing kidney suggests the inheritance of the branching nephridial pattern, but the cortex of the kidney develops later than the rest and has an independent blood-supply (Trueta, Barclay, Daniel, Franklin, and Prichard⁵⁷), thus perhaps suggesting a separate origin. The exact homology of the kidney structures with those of the adrenals and gonads must therefore remain doubtful.

In parenthesis, it may be suggested at this point that the meaning of the term homology is in need of some clarification. Many structures may become similar in character, though developing at different stages, simply because they arise from tissue which has at some earlier stage acquired certain characteristic biochemical and physiological properties

and particular patterns of behaviour, and because such tissue is in consequence more likely to develop in some directions rather than in others. In other words, the origin of the homology and similarity between organs may be much earlier and farther back in the evolutionary scale than is often supposed. For example, a given structure with properties similar to those of nephridia may be not strictly homologous with a particular nephridium, but it may be derived from tissue capable of nephridial development, and thus show characteristics in common with nephridia without itself having necessarily been derived from any one particular nephridium. It might then be described as showing general homology with nephridia without particular homology with any one nephridium.

As already mentioned, the zona glomerulosa of the adrenal cortex develops by the cells of the coelomic epithelium sinking into the underlying tissue, and at one stage of development vesicles are formed among the cells (Gruenwald^{38,39}), strongly suggesting atavism to the coelomostomes, and emphasizing the parallelism with the developing ovary, where Pflüger's egg tubes and the early follicles suggest the same origin. As in the ovary, also, cells may continue to join the zona glomerulosa from the peritoneal epithelium till quite late in life in certain species (Gruenwald³⁹). In fact, there is some evidence for a tendency in the adrenal cortex for the cells to move centrally, away from the coelomic cavity, till they degenerate on the borders of the medulla (Wotton and Zwemer⁶³; Hoerr⁴²; Bennett¹⁴). It is also noteworthy in this connexion that in grafts of adrenal cortical tissue (Williams⁶²), those taken from the outer zones will regenerate all the cortical tissue layers, but those from the zona fasciculata and zona reticulata tend to degenerate.

Just as the zona glomerulosa and the follicular layer of the ovary seem to be obvious homologues, the sex cords of the gonads and their derivatives are closely paralleled by the zona fasciculata of the adrenal, while the rete testis and rete ovarii have their counterpart in the zona reticulata of the adrenal. Like the rete ovarii the zona reticulata, or more strictly the X-zone, degenerates as the animal matures (see Text-fig. 27).

These parallels in morphological behaviour have their counterpart in cell structure and, more particularly, in cell metabolism, and from this point of view the cells of the adrenal cortex may be studied in more detail and their peculiarities discussed in relation to the probable origin of the gland.

There is one outstanding property which is shared to a greater or less extent by the cells of the adrenal cortex and the cells in the gonads. This is best described at present as their special lipid metabolism. Of all the tissues in the body, those under discussion are characterized by the storage of sterols and other lipoid substances, and by the secretion of sterols into the blood-stream, where they act as hormones. It is

interesting to note also that the fat-body in amphibia seems to belong to the same system, and the causation of this lipoid metabolism may initially have concerned the nutrition of the ova and sperm, and it may well be the remains of the characteristic yolk storage by the type B blastomeres from which the coelomic cells are in part derived.

From the adrenal cortex there have been extracted two main groups of physiologically active steroids. One group, exemplified by desoxycorticosterone, acts primarily on the salt balance of the body and regulates the excretion of Na and Cl by the kidney; the other, of which corticosterone and cortisone are examples, affects carbohydrate metabolism and the distribution of glucose. In addition many other steroids, including the sex hormones, androsterone, progesterone and oestrone, have been obtained from the adrenal cortex, and these last may in part be responsible for the abnormal sexual changes which, like virilism in women, are correlated with hypertrophy or malfunction of the adrenal cortex, and particularly of its inner zones. There is some evidence to suggest that the cells of the zona glomerulosa mostly produce the salt regulators while the carbohydrate regulators derive from the zona fasciculata. The fact that the main hormones of the adrenal cortex, while closely similar in structure and origin to the sex hormones, are primarily concerned with the regulation of the composition of body fluids, is of great interest in view of the probable phylogenetic relationship with coelomoduct or mixonephridial tissues which in more primitive animals must have had a precisely similar function. The recent observation of Bahl⁶ that the coelomic fluid of earthworms contains no glucose is probably significant in this connexion as indicating the ability of the coelomic epithelium or some part of it to regulate the distribution of glucose.

The distribution of lipids in the adrenal cortex is a matter of considerable uncertainty and variability, and their complete composition is far from being determined. One or two features, however, stand out clearly. The lipids of the zona glomerulosa behave independently of those in the zona fasciculata. For example, castration in rats leads to a general increase in the stainable lipids of the adrenal cortex, perhaps in an attempt to compensate for the loss of the sterols which are usually produced by the testes. Subsequent administration of testosterone or androsterone will then deplete the zona fasciculata and zona reticulata almost entirely but will leave the zona glomerulosa laden with sudanophilic granules (Hall and Korenschevsky⁴⁰) (Plate 1, Figs. 1-4). On the other hand, the zona glomerulosa may be emptied of its lipids by administration of desoxycorticosterone (Greep and Deane³⁶). Hypophysectomy (Deane and Greep²¹) also causes the lipids to disappear from the zona fasciculata and leaves those of the zona glomerulosa intact. Administration of corticotropic hormone causes deposition of lipids in the

zona fasciculata, but gonadotropic hormone may entirely deplete this zone (Carreyett, Golla, and Reiss¹⁸).

The lipids are clearly of several types and are not all steroids, though their composition and distribution is much in need of investigation. Crude measurements of weight or observations on the presence or absence of sudanophil granules are therefore of very little use in investigations of the functional changes in the cortical cells. All the particles which stain with Sudan III or Sudan black, or which blacken with osmic acid, are certainly not steroids, though there are reasons to believe that these substances are sometimes abundant in the adrenal cortex. The following table (Table II) outlines some of the main methods which have

TABLE II. *Staining Reactions used in the Detection of Lipids*

Sudan III and Sudan IV. Triglycerides in particular; less soluble in other lipids (Baker⁸).

Sudan Black. Stains most lipids in the cell. More soluble in lipids other than triglycerides.

Nile-blue Sulphate. Negative reaction (blue); saturated glycerides, fatty acids, and lipins.

Positive reaction (red); unsaturated glyceride, olein.

(N.B. There is also metachromatic staining of mucin (Cowdry¹⁹).)

Schultze's Modification of Liebermann's Method. A dark blue or reddish-purple colour develops and changes to green if a section containing cholesterol and its esters is treated with iron alum and then with a mixture of equal parts of concentrated sulphuric acid and glacial acetic acid (Lison⁴⁵).

Smith-Dietrich Test. Treatment with potassium bichromate, followed by staining with Kultschitsky's haematoxylin and differentiation with borax-ferrocyanide causes lipins to appear blue-black or grey-black, though lecithin alone may escape detection (Baker⁷; Lison⁴⁵).

Ciaccio's Methods. 1. Treatment with potassium bichromate renders certain lipids insoluble but stainable with Sudan III, while others are dissolved in the making of paraffin sections. Lipins and mixtures of cholesterol or cholesterolides with oleic acid are made visible.

2. Method 1 is preceded by an acetone extraction. Phospho- and galactolipins only are stained (Lison⁴⁵).

Digitonine Test (Windaus's method). Free cholesterol.

Phenyl Hydrazine Reaction (Pechet's modification). Corticosterones (Yoffey and Baxter⁶⁴).

Osmic Acid. Not specific.

Plasmal Reaction. Ketosteroids and aldehydes of fatty acids (Lison⁴⁵).

Acid Haematein Reaction. Positive after pyridine extraction indicates lipins (Baker⁷).

Birefringence. Free cholesterol. Glycerides and fatty acids may be birefringent after treatment with formalin. Cholesterolides may be birefringent at low temperatures.

been applied to the study of the cells of the adrenal cortex and the possible meaning to be attached to the findings. There is clearly room for greater precision.

The lipid content of the interstitial cells of the testis and ovary is also

very high and again very variable. For example, the cells of the membrana granulosa, before the follicle ruptures, are mostly small and without much inclusion material in the cytoplasm, but as soon as the follicle is ruptured the cells hypertrophy and become laden with lipids. The liberation of oestrogens by the follicle cells and of progesterone by the luteal cells is of course well known, but the parts played by the granulosa cells and the cells of the theca interna in the formation of these hormones is by no means clear. The interstitial cells of the testis are generally sudanophil and are the seat of origin of the androgenic sterols.

At first sight the kidney does not seem to fit into this picture of lipid metabolism, but in some species, as, for example, the cat and the dog, lipid material is often stored in large quantities in the proximal convoluted tubules and the tubuli recti. In the cat it is nearly all in the convoluted tubules; in the dog these are devoid of fat, but the tubuli recti are laden (Foote and Graafflin³⁰). The nature of the lipid has not been determined, but it is said to vary in amount quite independently of the amount of other storage fats in the body (Modell⁵²). Gérard and Cordier³² have called attention to the fact that cholesterol, when introduced into the body cavity of urodeles, may be segregated by the cells in a particular region of the proximal convoluted tubule of the kidney (mesonephros), and they have also observed that the nephridial funnels of earthworms show the same property. It is clear, therefore, that there is evidence for the inheritance of some special lipid metabolism by the cells of the kidney, and also for the functional similarity between nephridiostome and coelomostome.

The probability that many of the lipids in the 'coelomoduct-derived' tissues are lipins, and particularly phospholipins, as judged by the acid haematein reaction and by tests with Nile-blue, may be connected with the presence of phosphatase in the tissues, though in the kidney other functions have been assigned to this enzyme. When tested with glycerophosphate as substrate, as in the original Gomori technique (Gomori³³), the distribution of alkaline phosphatase in the cells of the urinogenital system is very variable from species to species and in the different organs. The proximal convoluted tubules of the kidney are laden with phosphatase in all species examined. The theca interna of the ovarian follicle is frequently positive to the test, and the corpus luteum may also contain many positive cells. In the testis the cells of the seminiferous tubules give a positive reaction, but the interstitial cells are negative. In the adrenal cortex the zona glomerulosa is nearly always negative, except in the guinea-pig, while the other zones, particularly those towards the centre of the gland, are sometimes positive and sometimes negative (Plate 1, Figs. 5-10).

These results should, however, be treated with caution, because it has been shown (Dempsey and Wislocki²⁴) that there may be several alkaline

phosphatases, each specific for a particular substrate, e.g. nucleic acids, glycerophosphates, hexose phosphates, &c., and the phosphatase distribution in the adrenals and sex glands is in need of re-investigation from this point of view.

When considered either as derivatives of coelomoducts with ciliated coelomostomes or of nephridiostomes, the cells of these organs may be expected to show one or two atavistic features. This they seem to do, for the brush border of the cells of the proximal convoluted tubules of the kidney may well be the expression of a past history of the formation of cilia. Moreover, the proximal convoluted tubules have the capacity for storing vital dyes and other particulate matter (athrocytosis), different substances being picked up in different places along the length of the tubule. In view of the possible relationship between the cells of the kidney tubule and those of the adrenal cortex the observation, already noted, that cholesterol is picked up mainly by the cells in the middle region of the tube (Gérard and Cordier³²) is of some interest. The zona glomerulosa of the adrenal cortex also picks up such vital dyes as trypan blue (Baxter¹³). Both in the kidney and in the adrenal cortex the dyes are stored as fine particles and not diffusely as by the typical histiocytes. Some of the cells of the membrana granulosa of the ovary show similar properties, and so do some of the interstitial cells in the testis, at least in certain animals (Rasmussen⁵⁵).

In several species of polychaete worms (*Hesionidae*, *Glyceridae*, and *Nereidae*) the coelomic funnel has a phagocytic epithelium and free phagocytes are often found in the cavity. So-called phagocytal organs are formed, and the genital funnel may lose its original function of conveying the germ cells to the outside, and be concerned entirely with 'cleaning up' the coelomic cavity (Goodrich³⁴).

Yellow lipochrome pigmentation, in which carotin and xanthophyll have been identified, is a common feature of the urinogenital tissues. The adrenal cortex, the kidney cortex, some of the cells of the corpus luteum, as well as the interstitial cells of the ovarian stroma and some of the interstitial cells of the testis, all contain visible amounts of yellow pigment. The distribution in the corpus luteum and among the interstitial cells of the testis is similar in that only certain cells contain extensive amounts of the pigment, while other cells are almost free of it. The pigmented cells often occur in groups. Similar pigments, of course, occur in many other epithelial tissues, but pigmentation is certainly a very conspicuous characteristic of the cells under consideration. It may be accidental, and may simply depend on the fact that the pigments are soluble in the lipids of the cells, and so collect there in much the same way as Sudan III dissolves in the fat in the usual test for lipids. Other fat depots often contain fat which is quite as heavily pigmented as is that in the cells of the urinogenital system.

Bahl⁶ has recently reported that in the nephridia of certain worms the cells may contain large amounts of haemochromogens. An investigation of the urinogenital tissues of vertebrates along these lines might prove of interest.

Another characteristic histological property common to several groups of cells in the urinogenital system is the fuchsinophil quality of the cells. Fuchsinophil cells occur in the inner zone of the adrenal cortex, particularly the X-zone, in the interstitial cells of the testis, in the corpus luteum, and in the convoluted tubules of the kidney, particularly in the young animal. Once again, however, it is necessary to stress the non-specificity of this staining, and until the basis for it can be firmly established it may mean very little. On the other hand, there is clearly a functional relationship between the X-zone of the adrenal cortex and the gonads; this fuchsinophilia may therefore be an index of the common function. The plasmal reaction which is given by ketosteroids and aldehydes of fatty acids is given by many of the same tissues, and these also stain heavily in iron haematoxylin preparations (Bennett¹⁴; Hoerr⁴²; Lobban⁴⁶). In the mouse embryo there is a well-developed X-zone, which in the male disappears at puberty. In the female, the zone persists after puberty but retrogresses slightly during each oestrous cycle, and is almost completely reduced during pregnancy (Deanesley²²) (Plate 2). It behaves as if it produced some essential commodity for the animal which became unnecessary when the sex glands became active in the production of the male sex hormones or of the related hormones of the corpus luteum. In the human adrenal cortex, the corresponding part, often known as the foetal cortex or androgenic zone, is the part which hypertrophies in cases of virilism in women, and under these conditions the cells of the ovarian stroma and of the corpus luteum show atrophic changes.

Castration in rats, as already mentioned, causes hypertrophy of the adrenal cortex and leads to an excessive deposit of fatty substances in the cortical tissue, a deposit which can be abolished by administration of testosterone (Hall and Korenschevsky⁴⁰). Furthermore, periodic changes in the adrenal cortex in relation to the oestrous cycle occur in the female rat (Anderson and Kennedy¹); and finally, adrenal tissue may start to develop in the stroma of the ovary when the adrenals have been removed (Groat³⁷), and similar tissue sometimes occurs in the testis.

Such a list of mutual interactions between testis, ovary, and adrenals could be still further lengthened, but sufficient has been said to make it clear that these tissues should all be considered together as part of one system, and the kidney should also be included, though perhaps less intimately, within this system. Ontogeny emphasizes the fact that all these tissues are derivatives of the coelomic epithelium and its immediately associated mesodermal and mesenchymal elements, and,

therefore, that they may be expected to exhibit family resemblances. Phylogeny adds to the picture by showing that they are probably derivatives of ancient coelomic canals (using this term in its broad sense of tubes connecting the coelom with the outside) which combined the functions of leading the sex cells to the exterior and of regulating the composition of the body fluids.

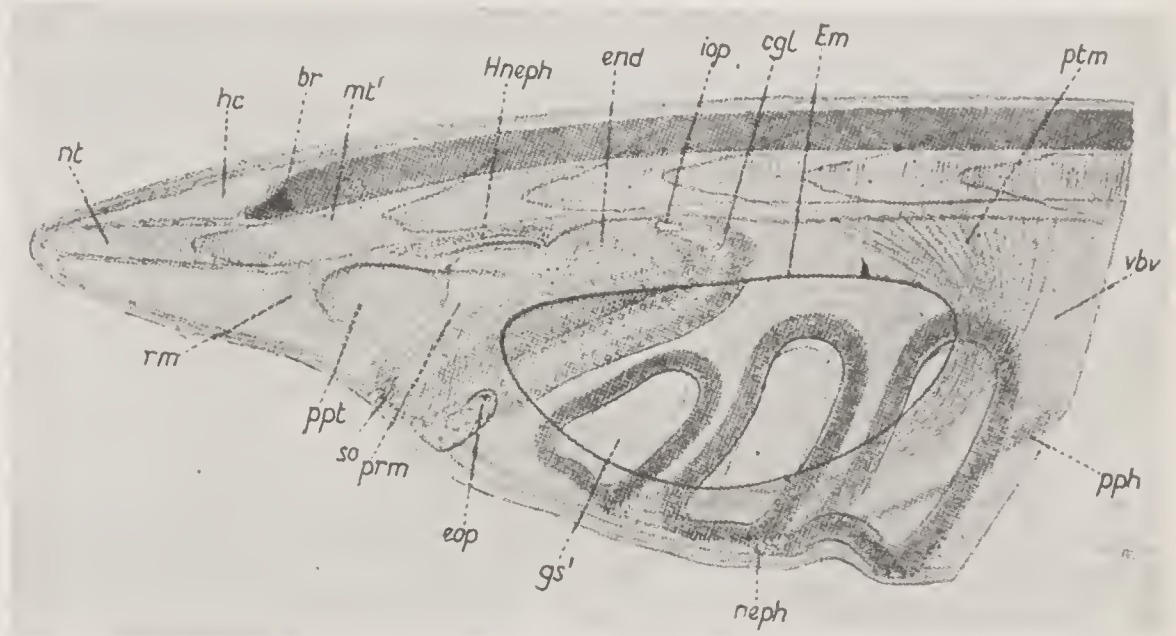
The phylogenetic approach may have another contribution to make to the picture, for there is one further unifying feature between these coelomoduct derivatives; they are all under the thumb of the pituitary gland; and their own activities, in their turn, largely determine the direction in which this hypophysial thumb twiddles. It is pertinent, therefore, to inquire into the nature of these connexions and interactions, and into the mechanisms by which they are brought about.

If the tissues in question, i.e. the urinogenital tissues, are all derivatives from the common system of coelomoducts, it is probable that they should all be affected to a greater or less extent by any agent which affected one of them. The reaction might not always be the same—in fact, it almost certainly would not be, since the cells come to lie in different environments and have evolved, more or less, in different directions—but it is reasonable to expect some sort of reaction in response to a common stimulus or to a stimulus which is effective on one member of the family. How, then, does the pituitary become involved?

The answer to this question is probably to be found in the solutions to two other problems. The first concerns the nature of the relationship between coelomoducts and nephridia and their representatives in the vertebrate body, and the second concerns the phylogenetic origin of the hypophysis. Unfortunately, those animals from which an answer to the second question might have been expected, namely, the direct ancestors of the vertebrates, have chosen to become extinct and to leave no trace behind them, so that the approach has necessarily to be indirect.

It has been argued that nephridia were originally segmental tubules leading from the exterior and ending blindly in flame cells, or else opening into the coelomic cavity by way of nephridiostomes. Coelomoducts, in the restricted sense of the word, seem also to have been originally segmental, arising as outgrowths of the segmental coelomic cavities. What, then, has happened to these two segmentally arranged and separate systems in the vertebrates, and what is their fate in the different regions of the body? These are the questions which need to be solved. If, as Goodrich supposed, the urinogenital system of vertebrates is derived entirely from coelomoducts, then we must suppose that the nephridial system, which is probably phylogenetically older than the coelomoduct system, has disappeared without trace, and we must presume that the cells which once formed it no longer develop in the embryo, or else that they degenerate at an early age. For such a thing to happen to the

whole system seems a little unlikely though, of course, not impossible. There is only one system of tubules in *Peripatus* (see Text-fig. 21), so something of the same sort has happened in the evolution of this creature, and it may be that this event will prove the key to the problem in relation to the vertebrates. Although no invagination from the epidermis is observed in the formation of the segmental tubes in *Peripatus*, nephridioblasts may, as suggested for the archinephric duct in the



TEXT-FIG. 28. Left-side view of anterior region of larval *Amphioxus*.

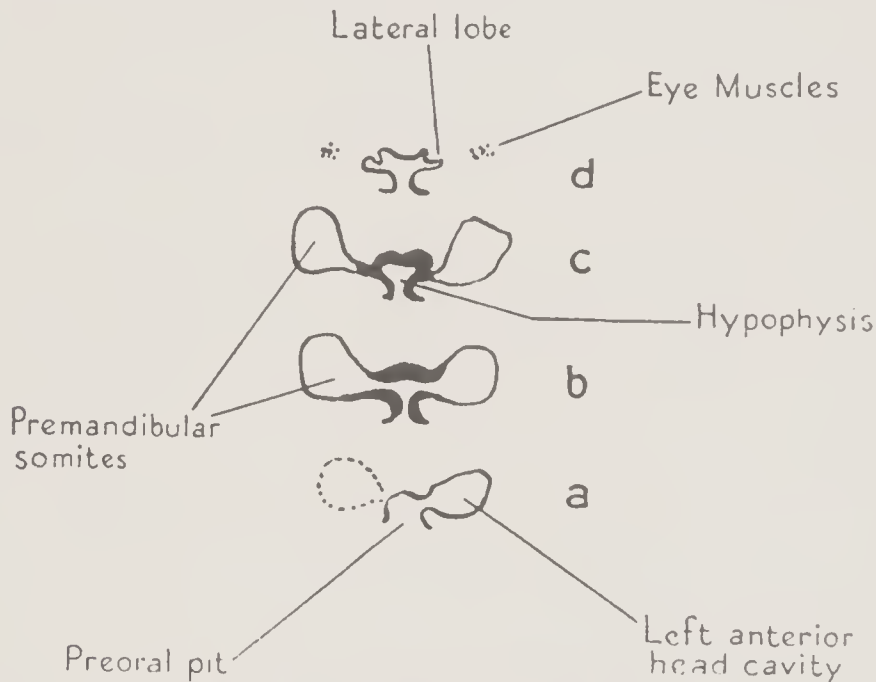
br. brain; cgl. club-shaped gland; Em. dorsal edge of mouth; end. endostyle; eop. external opening of gland; gs'. first primary gill-slit; hc. head cavity; Hneph. Hatschek's nephridium; iop. internal opening of gland; mt'. first myotome; neph. nephridium; nt. notochord; pph. peripharyngeal ciliated tract; ppt. preoral pit; prm. preoral muscle; ptm. postoral muscle; rm. rostral muscle; so. preoral sense-organ; vbv. ventral blood-vessel. (From Goodrich³⁵.)

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vertebrate, be involved in the formation of what is usually regarded as a coelomoduct, on the grounds that the primary outgrowth is from the coelomic mesoderm. In this sense the segmental tubes would be strictly mixonephridia, not coelomoducts, but the nephridial rudiment is precociously segregated, perhaps as individual cells, into the mesenchyme, and does not appear by invagination of a definite structure from the ectoderm. However, the case of *Peripatus* is probably to be considered as one of parallel evolution and not necessarily relevant to the problem in the vertebrates, except in so far as it shows a type of behaviour which may develop from a primitive nephridial and coelomic pattern.

While nephridia in most worms tend to be segmental throughout the length of the worm, coelomostomes and genital pores are less widely distributed, and the anterior segments may not possess them. In *Amphioxus* nephridia are well developed, and the pre-oral pit, which later in development becomes Muller's organ, is considered to be the remnant of the nephridium of the first segment and, interestingly enough, it has for a time in the young animal an opening into the left head cavity,

a cavity which is presumably the coelomic space belonging to the first segment. The pre-oral pit is a ciliated depression apparently concerned with passing a current of fluid towards the mouth (Text-fig. 28). In the fully developed *Amphioxus* the corresponding organ contains several sorts of cells and consists essentially of a ciliated pit surrounding a deeper depression known as Hatschek's pit, which has long, rod-shaped ciliated cells in addition to those of the more usual ciliated type. The



TEXT-FIG. 29. Diagram illustrating the relations in transverse section between the hypophysis and premandibular somites in *a. Amphioxus*; *b. Torpedo*; *c. Duck*; and *d. other amniotes*. (After de Beer²³.)

comparison of the form of these cells with that of some solenocytes suggests itself on the basis of a nephridial origin for the two groups. Finally, there is another structural character of the organ which, in view of the possible function of the organ, is a point of great importance; there are copious blood-vessels on one side of the ciliated pit. Now the pre-oral pit has been suggested (de Beer²³) as the homologue of the primitive pituitary body, or rather of its non-nervous part. This idea receives support from the observation that in the development of the elasmobranch, *Torpedo*, Rathke's pocket, which is the stomodaeal evagination which gives rise to the anterior lobe of the pituitary, opens for a time by lateral diverticula into the cavity of the first somite. Similar diverticula are also seen in the duck (Text-fig. 29). These observations all suggest that the anterior lobe of the pituitary is a modification of the nephridial and (or) coelomoduct rudiment corresponding to the first segment (de Beer²³). The recent observation by Bacq and Florkin² that the so-called neuroglandular organ of the Ascidian, *Ciona*, contains oxytocic and melanophore-expanding principles raises the question of the relationship between this organ, possible coelomoducts or nephridial elements in Ascidians, and the pituitary complex.

If the pre-oral pit of *Amphioxus* is really an indication of the origin of the hypophysis (excluding the pars nervosa, which is clearly of neural origin), then several relevant facts emerge. In the first place, the tissue is of the same general type as the tissue which has become modified into the urinogenital system, for both are developed from the segmental coelomic canals, using the expression again in its broad sense to describe tubules opening from the coelom, i.e. cavities, to the exterior, without prejudice as to whether the tubes are nephridia, mixonephridia, or true coelomoducts. Secondly, on the balance of available evidence, the hypophysis would seem rather to be a derivative of a nephridial system, whereas much of the gonad tissue is more obviously of coelomoduct origin.

Within the urinogenital system it has been shown that the function of one part affects the activity of another. Sexual activity modifies the behaviour of the adrenal cortex, and vice versa. Desoxycorticosterone modifies the excretion of Na and Cl by the kidney tubules. Ovarian activity modifies the behaviour of the endometrium of the uterus. Clearly these effects need not be confined to the urinogenital system proper, but may well spread to other derivatives of those tissues from which the urinogenital system takes origin; under this heading the pituitary now becomes involved. It not only becomes involved but it may be expected to dominate the system, and for this reason: the nephridia and coelomoducts have, primarily or secondarily, the function of regulating the composition of the body fluid and coelomic fluid. It is they which must deal with changes in the salinity of the environment or with adapting the organism to the fluid intake. Now the pre-oral pit, Muller's wheel organ, and Hatschek's pit in *Amphioxus* have always been regarded as merely transporting food to the mouth, but it seems likely enough that in transporting the food they are in reality acting as 'sense organs' for the composition of the fluid, but sense organs of a peculiar type. The activity of such cells will be modified by any abnormality in the composition of the fluid bathing them, and messages may be sent by the blood, with which, as already stressed (p. 485), Muller's organ and Hatschek's pit are copiously supplied, to the rest of the body, and in particular to the other nephridial or coelomoduct tissue. Evolution has then ensured that those parts which respond effectively do so. Thus, if an aquatic animal similar to *Amphioxus* enters an environment of reduced salinity or lower osmotic pressure, water will tend to penetrate the body and greater excretion may be required from the nephridia, but at the same time Na and Cl will have to be retained; alternatively, high salinity of the external medium may mean that water has to be conserved by the body and Na and Cl excreted. The pituitary 'sense organ' in the form of the Hatschek's pit complex may cause the necessary modification of the more posterior

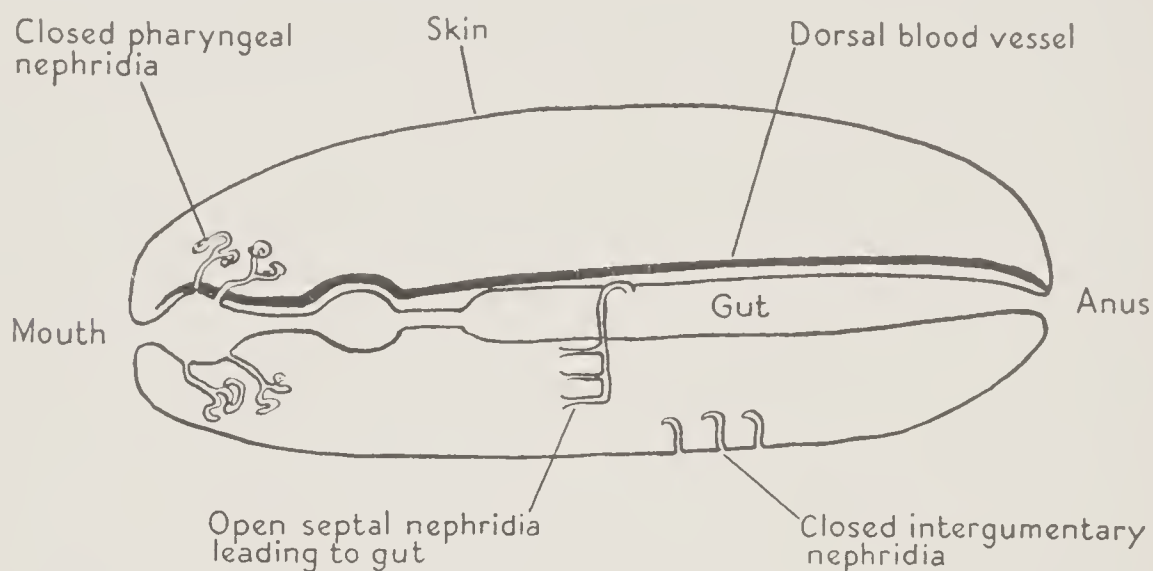
units by 'tasting' the water before it enters the body, and passing a hormone into the blood to cause the necessary modification of excretion. Furthermore, it is of vital necessity that the germ cells should not be shed into an unsuitable environment, so that control of the coelomoducts in relation to the composition of the environment would also be an obvious advantage.

In the higher animals, the vascular system becomes much more important as the unifying system of the body than the coelomic fluid. Consequently, on the one hand the coelomic canals (coelomoducts or nephridia) come into more intimate contact with the vascular system with the formation of glomeruli, and, on the other, the 'sense organ' is removed from the surface of the animal and there is a further increase in its vascularity, with the formation of the 'secretory' part of the pituitary. From now onwards it is changes in the composition and osmotic pressure of the blood, rather than of the external environment, which affect this 'sense organ'. The changes of the external environment are meanwhile becoming more the concern of the 'special senses', for which special organs become evolved. The intimate relationship between the coelomoduct derivatives and the vascular system allows better control by means of the pituitary because regulation can be on a reciprocal basis, pituitary affecting urinogenital system and vice versa. The situation is comparable with the regulation of the oxygen content and of the hydrogen ion concentration of the blood by the respiratory centre and carotid body becoming sensitive to changes in blood composition.

Removal of the 'sense organ' from the periphery, with its consequent disadvantages caused by the loss of contact with the outside world, is compensated by establishing a connexion, probably largely vascular, between the hypothalamic part of the nervous system and the anterior pituitary, and the development of osmo-regulation via the supra-optic nuclei, and the neurohypophysis is a further logical development (Verny⁵⁹). Interestingly enough, this move in the anterior end of the body, by which secretory and neural tissue are brought together, has its counterpart posteriorly in the urinogenital complex in the union between the cells of the adrenal cortex and the modified sympathetic ganglion cells of the adrenal medulla.

In 1903, Gaskell³¹ suggested that the pituitary, the thyroids, the parathyroids, the thymus, the adrenals, and the urinogenital system were all the derivatives of the primitive coxal glands, as found in such creatures as *Peripatus*. A review of the activities of the pituitary clearly indicates that this list of organs includes those which are most affected by pituitary secretions and among which there are numerous mutual interactions. Of the pituitary effects, which at first sight seem not to fit this idea, the growth hormone may exert its effect by multiple action

on several organs rather than specifically on growing cells. Indeed, it appears to have no action on the latter when tested by experiments in tissue cultures. The effect of prolactin on the mammary gland may become explicable when more is known of the phylogenetic origin of this gland, and the same applies to the influence of the pituitary on the pancreas. In this connexion the ultimate fate of nephridia must be investigated, for, as Bahl has shown (see p. 456), nephridia may subdivide into numerous small nephridia, each opening to the surface in a manner



TEXT-FIG. 30. Diagrammatic representation of the body of *Pheretima posthuma*, indicating the three types of nephridia present. (After Bahl⁶.)

similar to the sweat glands of higher animals; and also in some worms nephridia become connected with the gut rather than opening on to the external surface, and one wonders what is the phylogenetic origin of the sweat glands, the follicles of Langerhans in the ammocoete larva, the pancreas, and the other glands of the alimentary canal. The various types of nephridial variant are seen in Text-fig. 30, which shows the arrangement in the Megascolecid worm, *Pheretima*. The question therefore arises as to whether mammary glands and pancreatic ducts are related to an earlier nephridial system, or is it merely that the action of the pituitary is on some primitive metabolic characteristic common to large families of cells? In another field oestrogens, for example, seem to alter the mitotic rate in numerous epithelia (Bullough¹⁷). Can these epithelia all be shown to have been derived from, say, type A blastomere or something of the sort?

Another question of importance in considering the evolution of this coelomic canal complex is the nature of the relationship between the activities of coelomoducts and nephridia when both these systems are present in one and the same animal. As far as excretion is concerned, do they combine together or do they work in opposite directions, the one balanced against the other? The answer to this question and the determination of the nephridial or coelomoduct origin of the various

tissues may well provide a key to the understanding of the nature of the relationships between the various parts of the system. If the hypophysis is nephridial in origin and the gonads and adrenals, or certain parts of them, are derived from coelomoducts, a balance of one system against the other would suggest itself. It may be pertinent to this question that pituitary hormones are proteins, while the gonads and adrenals utilize steroids.

The general trend of this argument may be summarized as follows. In the urinogenital system there is evidence that the activity of one type of cell may modify the activity of other cells within the group; there is evidence that nephridial tissue and coelomoduct tissue may both be involved in different parts of the urinogenital apparatus and associated organs; each tissue will inherit properties according to its previous history; there is evidence that the pituitary body, thymus, parathyroids, and perhaps thyroid may be involved in this system as derivatives of the earlier nephridio-coelomic canal system, and thus that their actions may be all linked together. Furthermore, the organs so far mentioned mainly involve the coelomic canals belonging to limited regions of the body, and it is important to investigate what has happened to the remainder. Gaskell has even suggested that the lymphoid tissue and such tissues as the carotid and coccygeal bodies may be involved, but the whole problem needs investigation. There is certainly something attractive about including the lymphoid tissue, in that the thymus, for example, is largely lymphoid, and its activities, together with those of the rest of the lymphoid tissue of the body, are under the influence of corticosterone. Moreover, attention has already been called to the fact that several of the urinogenital tissues show pronounced properties of athrocytosis, though not to quite the same extent as do the histiocytes; and, finally, the formation of lymphocytes as an abnormal function of the kidney is a well-established phenomenon.

iii. CONCLUSIONS

How, then, is this problem particularly concerned with cytology and cell physiology? The significance is this. Early in the history of animals a type of cell has been segregated from the surface epithelium and has been concerned with excretion, the movements of ions, and the preservation of the proper fluid balance of the body. This is the nephridioblast. It acquires a certain form of metabolism which separates it from its neighbours, even though, at that stage, it may not visibly differ from these. Henceforth all derivatives of that cell will 'inherit' something of that metabolism. Similarly, the coelomic epithelium is another tissue segregated at an early stage and possessing certain particular metabolic characteristics, though its original cellular composition is more complex and two or possibly more types of cell seem to be included in its make-up.

The actual shapes and morphology of the cells of these tissues are often of little importance as compared with the way in which the cells behave. Comparatively few major advances have been achieved in cytology and cell physiology by studies of the static visible aspects of cells alone. It is cell behaviour that is important. The Golgi apparatus, the mitochondria, the nuclear pattern, and even the shape of the cell, all alter with the functional condition of the cell, and it is these changes which constitute the outward aspect of cell behaviour, but to understand the nature of that behaviour it is necessary to know more about the inner constitution of the cell. It is necessary to know the physiological inter-relationships between the different cells in the body and, to investigate this, it is essential to understand the inherited characters which form the common property of each cell group and the manner in which each group has arisen in the evolution of the animal.

Epitheliocytes differ from mechanocytes, and these again from amoebocytes, in certain definite and characteristic ways, as shown in Table I, and there may be two types of epithelial behaviour. These differences depend on the essential nutrients, metabolic products, manner of locomotion, relationship with neighbouring cells, and many other properties, and it is likely that, once a cell has been differentiated into one of these groups, the behaviour of that cell will thereafter follow along the line characteristic of the group to which it belongs. Reversal may be possible, but it clearly involves a very radical change of behaviour and, in most cases, embryological differentiation is a more or less irreversible process. An epitheliocyte may change the form of its Golgi net or of its mitochondria from moment to moment, but its capacity for forming a membrane or solid tissue by adhering to its neighbours is one that it does not easily alter. Similarly, the steroid metabolism of the coelomic epithelial derivatives, the ability of nephridial cells to deal with ionic distribution (and the distribution of carbonic anhydrase among the different tissues may be of interest here), the adrenaline-melanin metabolism of the neural crest and related cells are all features of fundamental importance and are deep-seated in the make-up of the cells which possess them. Moreover, when these cells give rise to new types, the new types so formed will possess many of the characters of their parents. For example, in the early blastula of the sponge (*Clathrina blanca*) there is a clear differentiation into two types of cell, both present in the epithelial layer. Something has already happened to divide the cells into two types, and therefore there must be in the adult sponge two main families of cells, those derived from type A and those derived from type B; and every cell in each of those families will possess some of the characteristics of the original type from which it sprang. When type A cells leave the surface they become mechanocytes, and all the derivatives of these cells will have certain common properties which will be

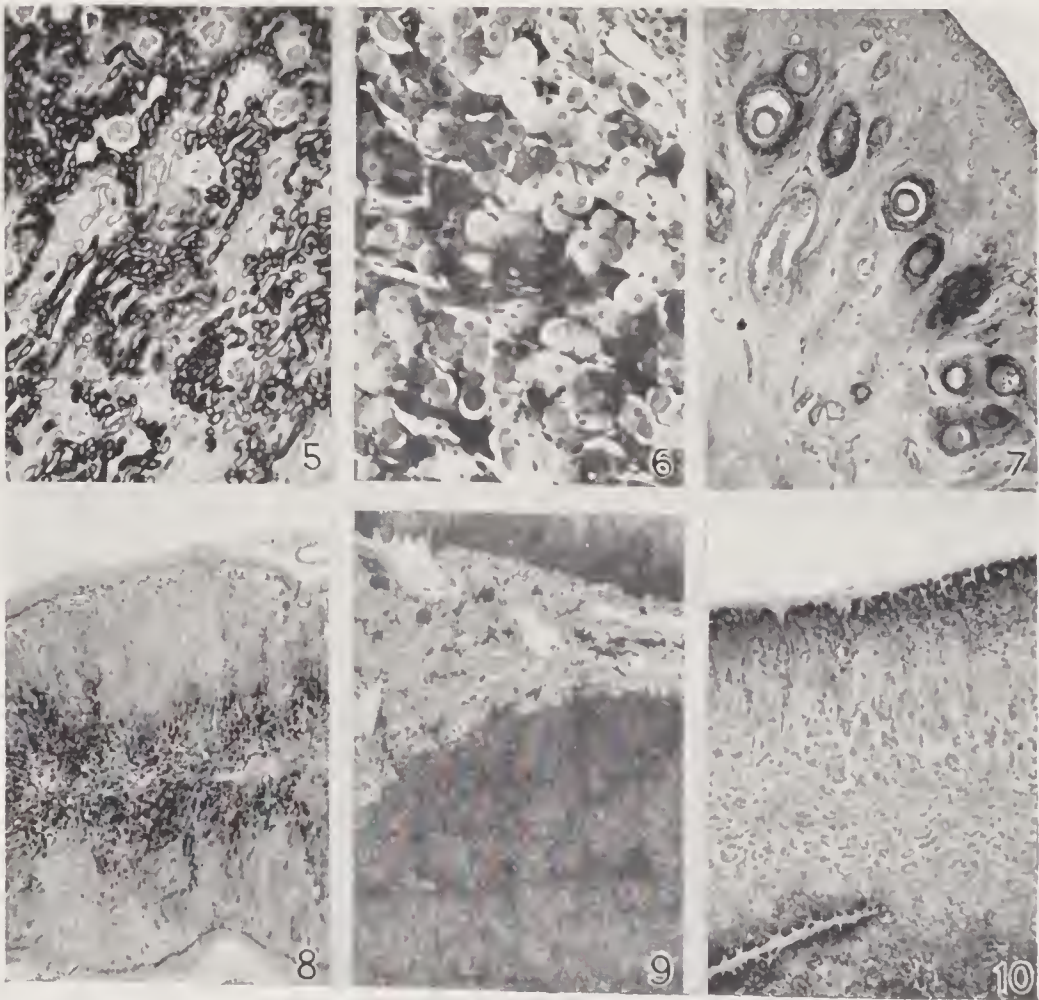
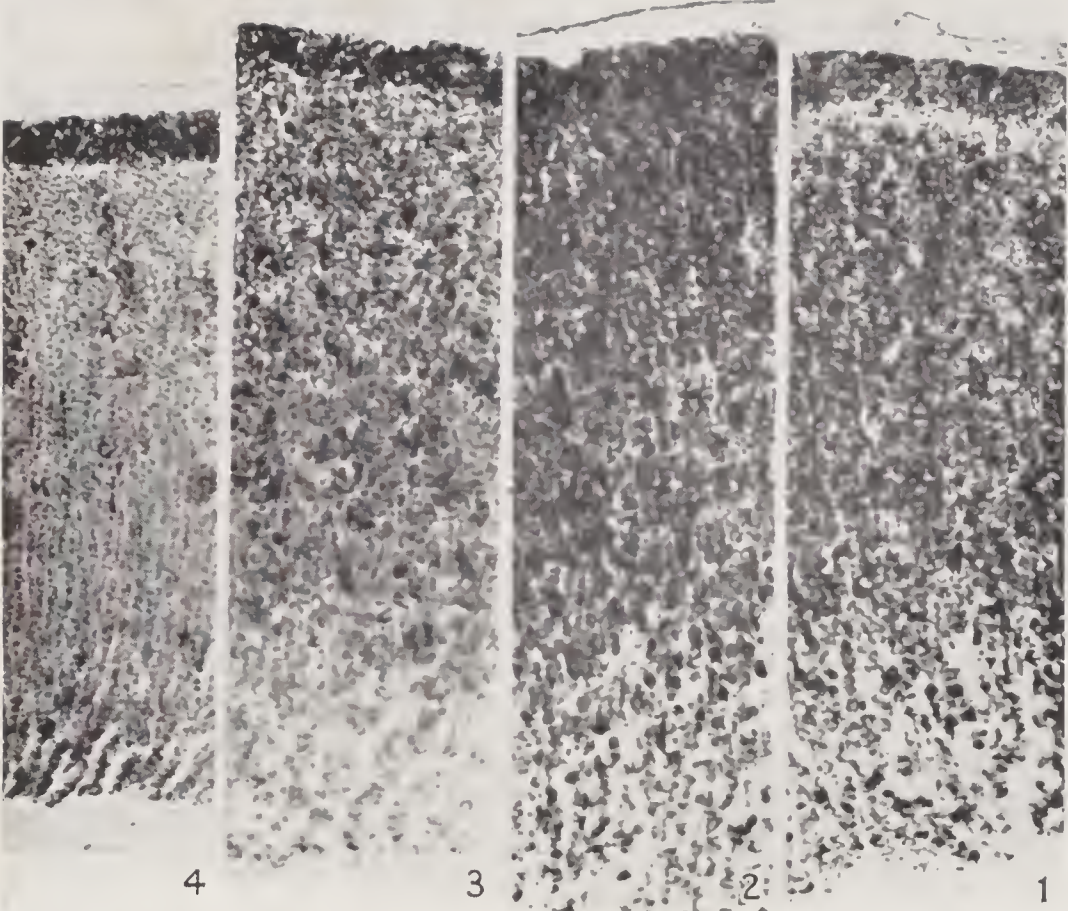
different in many ways from the properties of the amoebocytes which are derived from type B epitheliocytes. Let us suppose for the sake of argument that epitheliocytes of type A are stimulated by acetyl choline and inhibited by adrenaline, while the reverse might be true for the cells in type B; then, in so far as the cells in the organism responded at all to these stimuli, those belonging to one family would react in one way and those in the other family would respond in the opposite way. This is pure hypothesis, but may serve to illustrate the nature of the differences which might be expected between the cells belonging to the two groups.

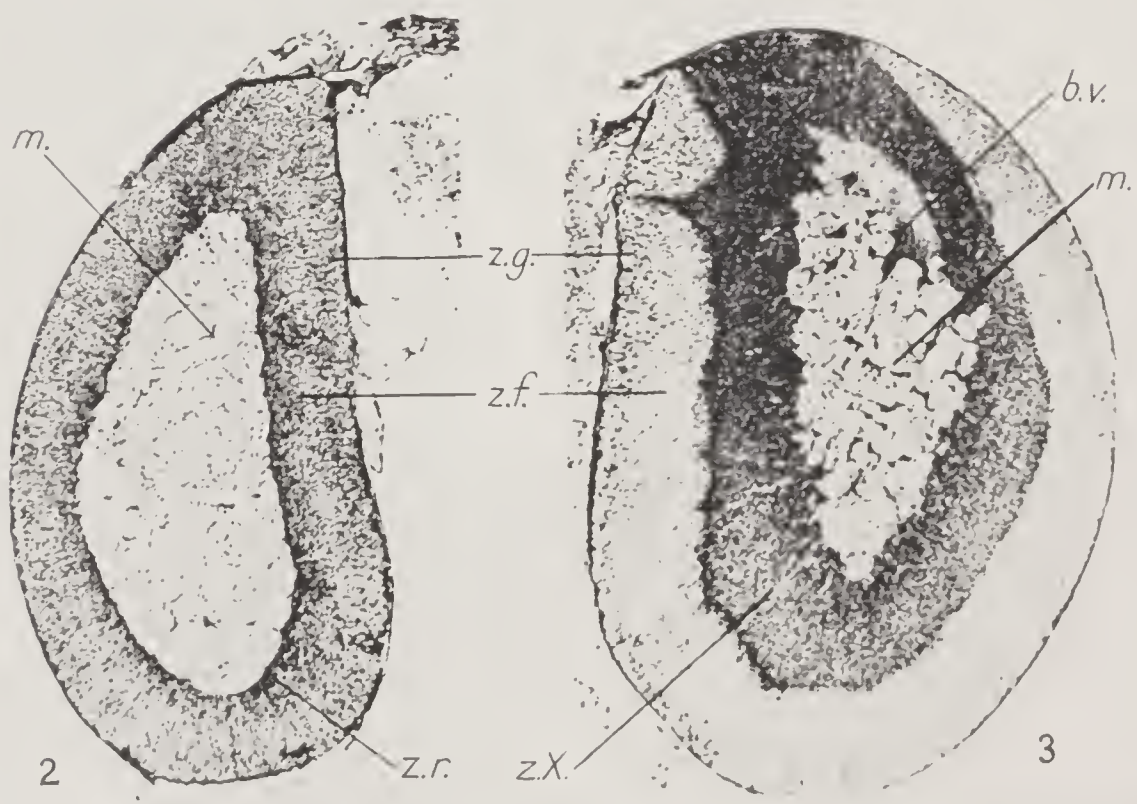
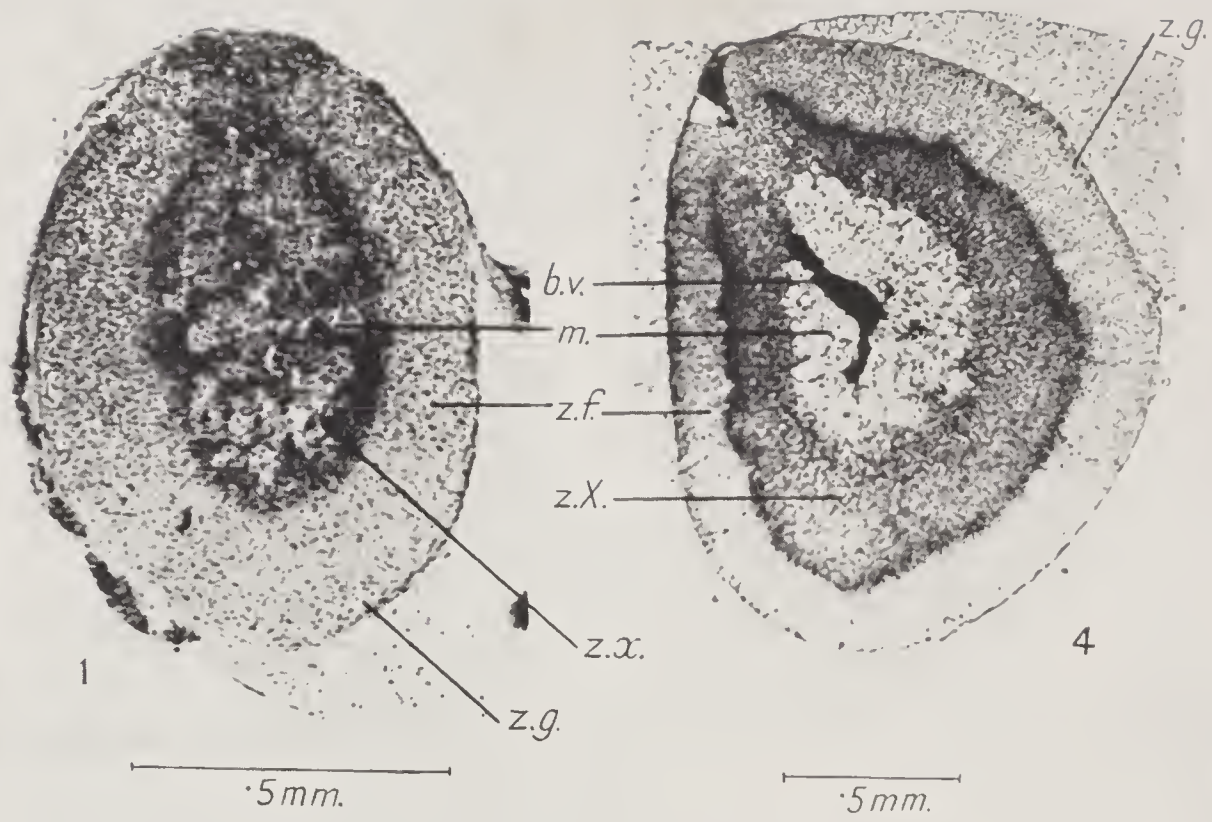
As evolution proceeds, the picture becomes more and more complex. Variations on the original themes occur from time to time, and in some instances the evolution may become convergent, but on the whole the proper perspective in cellular physiology can only be maintained when due weight is given to the data obtained by the study of the early history of the tissues under investigation. By early history is here meant the phylogenetic history even more than the ontogenetic, because the latter is often confused by alterations in the time scale of development by which some processes may be accelerated and others retarded, so that it is often difficult to decide which is the primary event. When the knowledge gained by these means can be combined with a more thorough knowledge of the cytochemical properties of cells, of specific cellular enzymes and metabolites, then cytology and cell physiology will stand on firmer ground than when these subjects depend on morphology and embryology only.

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DESCRIPTION OF PLATES

PLATE 1

FIGS. 1-4. Lipids in the adrenal cortex.

FIG. 1. Adrenal cortex of control rat; zona glomerulosa above, medulla below.

FIG. 2. Adrenal cortex of castrated rat. Note the high content of lipids, with little distinction between zona glomerulosa and zona fasciculata.

FIG. 3. Adrenal cortex of castrated rat injected with testosterone (0.033 mg. daily for 23 days). Note normal zona glomerulosa but impoverished zona fasciculata.

FIG. 4. Adrenal cortex of castrated rat injected with strong dose of androsterone (0.9 mg. daily for 47 days). Note the lipoid droplets still present in the zona glomerulosa, but their great reduction in the other layers.

(Figures from Hall and Korenschevsky⁴⁰.)

FIGS. 5-10. Alkaline phosphatase in urinogenital derivatives.

FIG. 5. Kidney of guinea-pig.

FIG. 6. Corpus luteum of sow, showing two types of cell.

FIG. 7. Ovary of dog.

FIG. 8. Human adrenal.

FIG. 9. Adrenal of rabbit.

FIG. 10. Adrenal of guinea-pig.

(Figures supplied by Dr. Gomori.)

PLATE 2

Functional changes in the adrenal gland.

FIG. 1. Section through adrenal of 19-days-old male; X-zone present.

FIG. 2. Section through adrenal of 12-weeks-old male; X-zone replaced by fibrous tissue towards medulla, or grading into the rest of the cortex.

FIG. 3. Section through adrenal of 12-weeks-old unmated female; X-zone well developed.

FIG. 4. Section through adrenal of 13-weeks-old male, 10 weeks after castration; the X-zone is of the female type.

(Figures from Deanesley²².)

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